The Development of Novel Apurinic/Apyrimidinic Endonuclease/Redox-factor 1 Inhibitors for the Treatment of Human Melanoma

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The Development of Novel Apurinic/Apyrimidinic Endonuclease/Redox-factor 1 Inhibitors for the Treatment of Human Melanoma

A Thesis by

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Submitted in partial fulfillment of the requirements for the degree of
Master of Pharmaceutical Sciences
December 2019

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December 2019
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ABSTRACT

The Development of Novel Apurinic/Apyrimidinic Endonuclease/Redox-factor 1 Inhibitors for the Treatment of Human Melanoma
by Bella (Banafsheh) Sharifi

Apurinic/apyrimidinic DNA repair endonuclease-1 (APE1), first recognized as an important DNA excision repair enzyme, is also known as Redox Factor-1 (Ref-1) involved in the activation of many nuclear transcription factors in both redox-dependent and independent manner. It has been well-documented that the overexpression of APE/Ref-1 contributes to the development of chemoresistance and is associated with tumor progression in many human malignancies [1]. Our previous study in melanoma demonstrated that the development of novel inhibitors targeting the redox regulation domain of APE/Ref-1 is a promising strategy for melanoma treatment. To date, limited successes have been reported in developing novel APE/Ref-1 inhibitors for cancer treatment. Utilizing a structure-based approach, our study identified and characterized small molecular inhibitors of APE/Ref-1. First, N-terminally truncated APE/Ref-1 protein lacking the first 40 amino acid residues (∆40APE-1\textsuperscript{wt}) was cloned into the pGEX-6P1 vector to express the GST-∆40APE-1\textsuperscript{wt} protein. After cleavage of GST-tag, the concentrated ∆40APE-1\textsuperscript{wt} protein was subjected to protein crystallization study. We have successfully diffracted ∆40APE-1\textsuperscript{wt} crystals and collected data with a resolution of 1.57Å. The crystal structure was further determined by molecular replacement in Molrep using the already available human APE-1 structure (PDB: 5CFG). For the first time, we observed the dimerization of APE/Ref-1 protein formed under oxidative conditions, which may contribute to the redox regulation of APE/Ref-1. Such structural transformation of APE/Ref-1 protein under distinct redox conditions may pave the way for future drug development and optimization. The binding affinity of the candidate compounds with ∆40APE-1\textsuperscript{wt} protein was also determined using Surface Plasmon Resonance (SPR), and the $Ki$
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# LIST OF ABBREVIATIONS

<table>
<thead>
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<tr>
<td>Δ40APE-1&lt;sup&gt;C138A&lt;/sup&gt;</td>
<td>Truncated APE/Ref-1 cysteine 138 mutated to alanine</td>
</tr>
<tr>
<td>Δ40APE-1&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>Truncated APE/Ref-1 at first 40 amino acid</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APE/Ref-1</td>
<td>Apurinic (apyrimidinic) endonuclease/ redox-factor-1</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant Response Element</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTB</td>
<td>Broad complex, Tramtrack, and Bric-a-Brac</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CM</td>
<td>Cutaneous melanoma</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>ECFC</td>
<td>Endothelial colony forming cell</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione Peroxidase</td>
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GSH  Reduced Glutathione
GSSG  Oxidized disulfide
GST  Glutathione S-transferases
H$_2$O$_2$  Hydrogen Peroxide
HEPES  (4-(2-hydroxyethyl)‐1-piperazineethanesulfonic acid)
HO-1  Heme oxygenase 1
IVRIVR  linker intervening region
Keap 1  Kelch-like ECH-associated protein 1
NF-κB  Nuclear factor-κB
NQO1  Quinone oxidoreductase 1
Nrf-2  Nuclear factor erythroid 2-related factor-2
P53  Tumor suppressor p53 gene
PBS  Phosphate-buffered saline
PEG  Polyethylene glycol
PIC  Protease inhibitor cocktail
RNS  Relative nitrogen species
ROS  Reactive oxygen species
RPM  Revolutions per minute
SDS  Sodium Dodecyl Sulfate
STAT3  Signal transducer and activator of transcription
UVR  Ultraviolet radiation
# LIST OF SYMBOLS

<table>
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<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>°C</td>
<td>Degree of Celsius</td>
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1 Chapter 1

1.1 Introduction

While the incidence of many of the most commonly diagnosed cancers in the United States (e.g., breast, colorectal and prostate cancer) have been declining for more than a decade, malignant melanoma remains one of the few cancer types in which the incidence continues to rise, raising substantial public health concerns [2]. Highly effective therapy is primarily limited to surgery, which occurs only when the diagnosis is achieved at the early stages of the disease; traditional chemotherapy is not highly effective in more advanced stages of melanoma with only 24.8% of patients with distant metastasis surviving over five years [3]. In recent years, there have been dramatic developments in the treatment of advanced cutaneous melanoma (CM) using revolutionary immunotherapy [4]. Although these powerful checkpoint inhibitors yield remarkable and durable responses in melanoma patients, they are mainly indicated for patients with advanced melanoma and disease progression following other treatments [5]. Furthermore, although such incredible progress in the treatment of melanoma has been made, the world-wide melanoma epidemic has not been slowed down. This disease continues to be an immense public health challenge, especially in the United States. Therefore, more novel and effective strategies blocking melanoma genesis and disease progression to the advanced stage are highly desired [6].

The aberrant signal pathways or mutant genes observed in cancer cells may be their “Achilles Heel,” which can be utilized as druggable targets for developing novel targeted therapy [7]. Driven by this strategy, our research is focused on identifying effective novel targets and approaches for
melanoma prevention and therapy, primarily through the extensive mechanistic studies of abnormal redox signal pathways identified in melanoma during disease progression.

APE/Ref-1, first recognized as a DNA endonuclease base excision repair (BER) enzyme, which plays an important role in redox signaling pathways via reduction of oxidized cysteine residues of many nuclear transcription factors (TFs), such as Nrf-2, NF-xb and AP-1, resulting in transcriptional activation of genes associated with cancer proliferation and progression [8]. Of note, abnormal expression of APE/Ref-1 is reported in many human malignancies, including melanoma. Our previous studies have shown that APE/Ref-1 plays an essential role in melanoma progression and the development of drug resistance. Targeting APE/Ref-1 using small molecular inhibitors is shown as a potent and effective strategy for melanoma therapy. A novel APE/Ref-1 inhibitor, APX3330 (known as E3330), exhibits promising pre-clinical anti-tumor activities and is currently studied in a clinical trial [8].

1.2 APE/Ref-1 function and role in melanoma development/drug resistance

Throughout the life cycle, organisms and cells undergo constant DNA endogenous and exogenous damages caused by respiration, replication, ultraviolet (UV) or ionizing radiation (IR). Depending on the severity of these DNA damages, cells can either arrest to overcome the damage or undergo apoptosis [9]. Human APE/Ref-1 consists of 318 amino acids, with a molecular weight of 36.5 kDa [10]. Further studies have shown that APE/Ref-1 contains three distinct functional domains. The first 25-33 N-terminal unstable structured amino acid sequences are responsible for nuclear localization signaling. Deletion study of the first 40 amino acids responsible for nuclear localization and loss of function showed that the truncated APE/Ref-1 fails to translocate to the nucleus. This domain is also called nuclear localization signal domain [11]. The second N-terminal
region amino acids are responsible for redox activity. This region is followed by the third domain at the C-terminal region, which is responsible for DNA repair activity. Studies showed that APE/Ref-1, functioning as an important endonuclease, effectively repairs oxidative DNA damage via the base excision repair pathway (BER) [11].

It has been well studied that APE/Ref-1-mediated signaling plays an important role in cancer development and progression. The expression of APE/Ref-1 is known to be highly sensitive to redox-status alterations. Our previous studies consistently demonstrated abnormally elevated nuclear APE/Ref-1 in human melanoma cells compared with normal melanocytes, which is associated with drug resistance and proliferation [12]. Cells exposure to elevated levels of ROS can regulate APE/Ref-1 activity and expression on both transcriptional and posttranscriptional levels [13]. Since melanoma cells exhibit abnormal intracellular redox status, the induction of APE/Ref-1 observed in tumor biopsies may be an adaptive response to the prolonged exposure of oxidative stress, which contributed to cell proliferation and disease progression [12].

Studies done in other human malignancies such as pancreatic and ovarian cancers have also demonstrated up-regulation of APE/Ref-1 secondary to oxidative DNA damage [11]. As a result, overexpression of APE/Ref-1 is associated with increased cell survival from chemotherapy and DNA-interactive drugs [11]. Taken together, APE/Ref-1 might be a promising druggable target to improve the outcomes of melanoma therapy. The critical role of APE/Ref-1 in promotion, progression, and drug resistance in melanoma and other types of tumors, states its target potency for development of new cancer therapeutic and preventive strategies [12]. Thus, in recent years, further efforts have been directed to identify, design, and develop small molecule inhibitors of APE/Ref-1 to improve cancer treatments [10].
As such, the ultimate goal of our study is to develop novel and potent inhibitors, which can selectively block the redox activity of APE/Ref-1.

1.3 APE/Ref-1-mediated redox regulation of nuclear transcription factors

Epidemiology studies showed that certain risk factors or environmental exposure, such as indoor tanning, atypical mole, fair skin type as well as excessive UV exposure at a young age are associated with increased risk of developing melanoma [10]. Melanin is the major pigment found in the hair, the epidermis, and the brain as well as other highly nerve-active areas such as retina and middle ear. It is a unique product of melanocyte cells and is responsible for skin protection from photochemical stress [12]. Melanin plays as a disposable buffer as it neutralizes upregulation of reactive oxygen species (ROS) generated by ultraviolet radiation. The mechanism by which melanin consumes oxygen and superoxide to produce hydrogen peroxide involves the organelle melanosome. As a result, melanosomes constantly become more structurally, and functionally disordered, which generates higher levels of ROS that increases hydrogen peroxide stress at melanoma cells [12]. Cells mechanism in response to peroxide stress have shown that normal melanocytes can efficiently abrogate the peroxide stress, while this ability is seriously impaired in melanoma cells, which evoking higher levels of reactive oxygen species (ROS).

Additionally, UVR has also been known to be associated with the development of skin malignancies, as it causes skin inflammation by releasing inflammation mediators like cytokines (1L-1 and 1L-6), which further generates great levels of ROS (i.e., H₂O₂) and induces oxidative stress. Elevated ROS induces various oxidative DNA damage such as a mutagenic lesion at 8-oxodihydro-2'-deoxyguanine (8-oxo-dG) which is directly repaired by the DNA base excision-repair
pathway [12]. In addition to environmental exposure cytotoxicity, organs encounter reactive oxidants from internal metabolism.

It has been well documented that APE/Ref-1 is highly sensitive to both intracellular and extracellular environments as it is an adaptive response to oxidative stress. Elevated levels of reactive oxygen species (ROS) generate not only direct oxidization of amino acid residues at the N-terminal domain of APE/Ref-1, but also increase subcellular localization of the protein [12]. APE/Ref-1 is an essential protein in protecting cells from ROS toxicity; however, overexpression or activation of APE/Ref-1 as an adaptive response to prolonged oxidative stress transforms the cellular signaling to proliferation and antiapoptotic phenotype [12]. The N-terminal redox function of APE/Ref-1 is responsible for the activation of many transcription factors, including nuclear factor-kB, HIF-α, and AP1, which have been well studied in many cancer cells. Therefore, APE/Ref-1 may play an essential role in protecting cells from oxidative stress not only through repairing oxidative DNA damage but also via regulating nuclear transcription factors and the transcription of their downstream target genes [14]. It has been proposed that one of the redox-regulation mechanisms of APE/Ref-1 is based on oxidation and reduction of the thiol group of cysteine residues [15]. According to the study, APE/Ref-1 might bind to thioredoxin, which serves as a very potent reducing reagent [14]. By binding with thioredoxin, oxidized APE/Ref-1 will be reduced, and the active form of APE/Ref-1 could bind to nuclear transcription factors. The other underlying redox-regulation mechanism of the APE/Ref-1 is proposed to be through redox regulation of many transcription factors (TFs) [10] with possible structural changes that are associated with distinct redox status [14]. The loss of purine bases during DNA division leads to the loss of potentially vital genetic information in cells. BER is the primary pathway to repair DNA damages and to overcome the mutagenic and cytotoxic effects caused by spontaneous oxidation,
non-enzymatic alkylation, and hydrolytic processes [11]. Apurinic/apyrimidinic endonuclease-1/Redox factor-1 (APE/Ref-1) is a multifunctional protein, which is known to be essential in base excision repair (BER) pathway signaling that is involved in DNA repair and nuclear redox regulation of many transcription factors [12]. In redox mechanism, APE/Ref-1 regulates activation of multiple cellular transcription factors including nuclear factor-κB (NF-κB) [16], signal transducer and activator of transcription (STAT3), activator protein-1 (AP-1), Nrf-2 (Nuclear factor erythroid 2-related factor-2).

APE/Ref-1 is one of the most promising proteins with the reduction-oxidation activity that can influence multiple cancer survival mechanisms, including growth, proliferation, metastasis, angiogenesis, and stress responses [17]. Studies on human melanoma cell lines have indicated the role of APE1/Ref-1 as an adaptive response to oxidative stress, cancer progression, chemoresistance, and increased morbidity [18]. The tumor microenvironment contains stromal cells such as fibroblasts, endothelial cells, and macrophages [18]. The high-density tumor-associated macrophages correlate to poor prognosis, cancer progression, and secretion of several inflammatory mediators, including TNF-α, interleukin-6 (IL-6), reactive oxygen species (ROS), and nitric oxide (NO) [19]. The intracellular redox status of APE/Ref-1 appears to be a key regulator of tumor cell growth [20], which makes the protein a promising candidate for anti-cancer therapeutics [21]. As previously mentioned, human cells are constantly exposed to oxidative and electrophilic chemicals both from endogenous and exogenous. Despite the important mechanism of reactive oxygen species (ROS) and relative nitrogen species (RNS) in cellular redox hemostasis, induced oxidative stress contributes to cell damage, chronic inflammation, and cancer initiation [22].
1.4 The role of Nrf-2 in redox regulation

Antioxidant response element (ARE) is a cis-regulatory element with specific DNA sequences at upstream regulatory regions of genes that are responsible for encoding detoxifying enzymes and cytoprotective proteins [23]. The antioxidant defense mechanism is found to be the major protective response in maintaining redox hemostatic through upregulation of various enzymes and cytoprotective compounds such as quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), glutathione S-transferase (GST), heme oxygenase-1 (HO-1) catalase and thioredoxin [22].

The three main cellular antioxidant responses, which found to be involved in maintaining the cellular redox balance are Kelch-like Ech-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf-2) and the antioxidant response element (ARE) [22]. Keap1 is constantly bound with Nrf-2 in cytoplasm through ubiquitin-dependent proteasomal maintaining cells growth at basal level. Under normal circumstances, Keap1 maintains cellular redox function of Nrf-2 by acting as inhibitor and preventing its localization to nuclear via 26S proteasomal pathway. In this mechanism, homodimerization of Keap 1 via its BTB to Neh2 domain of Nrf-2 (at ETGE and DLG motif) maintains the steady-state of Nrf-2 at basal level [22]. Studies have proposed that the Cys$^{273}$ and Cys$^{288}$ at the linker intervening region (IVR) along with Cys$^{151}$ at Broad complex, Tramtrack, and Bric-a-Brac (BTB) domain of Keap 1 play important role in maintaining Nrf-2 at basal level in the cytoplasm [24]. Upon oxidative stress, cysteine residues of Keap1 at BTB and IVR domain are covalently modified, leading in conformational changes of Keap1 dimerization [25]. As a result, Nrf-2 is escaped from Keap1-mediated degradation, translocate to the nucleus and activates ARE-dependent antioxidant gene expression such as HO-1, NQO1, and several glutathione S-transferase components to regulate and maintain redox homeostasis and cell survival via formation of the heterodimer with Maf protein [22]. Therefore, the redox balance of the cell is found to be
necessary in Keap1-Nrf-2-ARE antioxidant pathway gene expression [22]. Nrf-2 is a bZip (basic leucine Zipper) transcription factor that contains 605 amino acids with six conserved domains, Neh1 to Neh6. Neh1 domain contains bZip motif that is found to be necessary for heterodimerization with musculoaponeurotic fibrosarcoma (Maf) protein, allowing Nrf-2 to bind with ARE activating gene. The N-terminal, Neh2 domain negatively regulates Nrf-2 transcriptional activity by binding to Keap1 domain through DLG and ETGE motif. The Neh3 to Neh5 domains are responsible in mediation of Nrf-2 transactivation activity. Lastly, thought the Neh6 domain, Nrf-2 can be negatively regulated by Keap1-independent protein [22]. It has been proposed that among six cysteine residues of Nrf-2, Cys^{183} and Cys^{506} are found to be necessary in their antioxidant response [22]. Over the past few decades, many studies have highlighted the major redox regulation function of APE/Ref-1 in response to oxidative stress, which can lead to activation of Nrf-2 and its downstream target genes expression [26-28].

1.5 Development of novel APE/Ref-1 redox inhibitors

Cells are continuously subjected to reactive oxygen species produced by a normal metabolic process, and APE/Ref-1 protects cells from ROS toxicity within cells mechanism [29]. As mentioned earlier (1.2), APE/Ref-1 is also involved in response to elevated ROS caused by UVR exposure. The role of APE/Ref-1 in inflammation and oxidative damage by cause of sunburn and UVR exposure indicates interference with melanomagenesis by targeting APE/Ref-1 as a potential preventive strategy [14]. The redox effector factor of APE/Ref-1 was first recognized by its DNA base excision repair activity at its C-terminal domain, followed by its redox activity at N-terminal domain which exhibits distinct redox regulation activity that facilitate DNA binding activity of many transcription factors such as NF-kB [30], HIF-α [31], and AP-1 [32]. The APE/Ref-1 in redox and repair function has made them a potential druggable target in various human
pathologies. However, little success has been made over the past two decades on developing potent inhibitors targeting the DNA repair domain of APE/Ref-1. Therefore, major efforts have been directed to identify and synthesize inhibitors to block the redox activity of APE/Ref-1 [11].

Studies have shown that the redox regulation activity of APE/Ref-1 is remarkably reduced by either H$_2$O$_2$ oxidation or cysteine replacement by direct mutagenesis [33]. It has been proposed that APE/Ref-1 might regulate the binding activity of transcription factors via maintaining them at reduced state by reduced cysteine residues, specially Cys65 and Cys93 at its redox domain [34]. Studies have proposed that Cys65 serves as the nucleophilic residue in the reduction of disulfide bonds within reduced transcription factors by APE/Ref-1 [17].

Recently, high demand virtual screening (VS) of three-dimensional protein structure by chemical libraries has delivered rapid drug discovery development using docking strategy. The diverse compound screening ability of VS has brought numbers of success drug discoveries such as E3330 [12]. Luo et al. [35] reported a promising redox inhibitors of APE/Ref-1, [(2E)-3-[5-(2,3-dimethoxy-6-methyl-1,4- benzoquinone)]-2-nonyl-2-propenoic acid, also known as E3330. E3330 is a known promising clinically available APE1 redox-specific inhibitor, which has shown stimulation of lipopolysaccharide (LPS) macrophages [18]. The compound was first known as NF-kB inhibitor, but more studies revealed its novel mechanism by Matrigel assays and aortic ring formation assays [17]. In this mechanism, E3330 suppresses the secretion of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL-6) and IL-12 and inflammatory mediators nitric oxide (NO) as well as the LPS-stimulated cells [18]. The compound successfully inhibits APE/Ref-1 redox regulation function both in vitro and in vivo by blocking Endothelial colony forming cell (ECFC) angiogenesis [17]. Downregulation of the LPS-dependent expression
of inducible nitric oxide (iNOS) has also been indicated by E3330 inhibitor. Therefore, E3330 has been known as a potent candidate in inflammatory response suppression, which provides a novel therapeutic strategy for the inhibition of tumor-associated macrophages [18].

In our previous studies obtained by Yang et al. [12] group, a sophisticated computer program, ICM software developed by MolsSoft L.L.C [36, 37] was used to model a three-dimensional structure of APE/Ref-1. The software identified a potent druggable pocket located at the redox regulation domain of APE/Ref-1. Conducting extensive virtual docking of over 3-million compounds using the ICN database they identified potent lead compounds with successful docking into the N-terminal redox regulation domain of the APE/Ref-1. Using further cell-based bioactivity screening, our group successfully developed R21, our lead APE/Ref-1 inhibitor with promising anti-melanoma activity [38]. Our group further synthesized R21-OH, one of the analogs of R21, with improved water solubility compared to its parental compound, R21. Our promising compound, R21-OH, exhibited significant anti-melanoma activities at very low concentration (<5 µM) compared to the other well studied APE/Ref-1 inhibitors such as E3330 and E2009. One of the objectives of this study is to utilize a structure-based strategy using R21-OH as our lead promising inhibitor to determine the binding characteristics of novel APE/Ref-1 inhibitors with the protein, which will shed lights on design and development of more potent small molecular binders. In addition, in our previous studies by Yang et al. [12] group, the two potent compounds, 2010-05 and SY598-21, also exhibited promising APE/Ref-1 redox inhibitory and anti-tumor activity in vitro. The previous MTT results of our promising compound exhibited high cytotoxicity to human melanoma cell line, A375, with improved water solubility compared to E3330. Taking these candidates as lead compounds, we will determine the binding characteristics of APE/Ref-1 in order to block the protein redox activity.
Studies on nuclear transcription factors, have revealed mediated signaling of Nrf-2 on a set of antioxidant proteins via binding to antioxidant response elements [39]. In addition to the DNA repair activity of APE/Ref-1, the redox regulation of the protein via cysteine residues has shown the significant role in reducing transcription factors such as Nrf-2, enhancing their binding to target gene promoter [40]. Our previous studies on co-immunoprecipitation assays revealed a direct binding interaction between APE/Ref-1 and Nrf-2. In our study, we will further determine if the binding of APE/Ref-1 with Nrf-2 is redox-dependent or not.

Elevated Ref-1 levels and its abnormal cellular location is associated with many human tumors including brain, cervical, pediatric rhabdomyosarcomas, prostate, epithelial ovarian cancers, and germ cell tumors [41-45]. Upregulation of APE/Ref-1 protects cells from various pro-apoptosis stimuli including oxidative stresses, chemotherapeutic drugs and radiation treatment [46-48]. Down-regulation of APE/Ref-1 expression is associated with sensitization of cells to induced apoptosis both in vitro [49, 50] and in vivo [51, 52]. These results suggest that drugs targeting APE/Ref-1 and especially the Ref-1 activity might be useful in combating cancer and, indeed, compounds impairing APE/Ref-1-mediated signaling have enhanced therapeutic efficacies in many human tumors [53, 54]. Moreover, pro-apoptotic effects of APE/Ref-1 are associated with p53 regulation [55-57]. In addition, elevated APE/Ref-1 is closely associated with increased metastatic potential owing to redox activation of nuclear transcription factors such as Nrf-2, STAT3, NF-κB, and AP-1 [58-62]. Recent genome-wide analyses and proteomic studies revealed an important role of APE/Ref-1 in regulating many biological processes, such as mitochondrial function, cytoskeletal structure, and rRNA quality control [63-65]. It thus is clear that APE/Ref-1 is an attractive target for the development of new cancer preventive and therapeutic strategies [43, 65, 66]. The most potent APE/Ref-1 inhibitor developed by our group using structure-based
approach showed promising anti-melanoma activities both in vitro and in vivo. Taking this candidate as our lead compound, we aim to determine the binding characteristics of APE. Ref-1 with our novel inhibitor and further develop more novel APE/Ref-1 inhibitors for human melanoma therapy. We also aim to conduct experimental strategies to understand the direct interaction mechanism and the redox regulation of APE/Ref-1 protein to nuclear transcription factors.
Chapter 2

2.1 Methodology

2.1.1 Overexpression of Δ40APE-1<sup>wt</sup> protein

The cDNA encoding a N-terminal truncated APE-1<sup>wt</sup> protein lacking the first 40 amino acid residues (Δ40APE-1<sup>wt</sup>; 31.3 kDa) was cloned into the pGEX-6P1 vector. The plasmid was then transformed into the one shot® BL21 Star™ (DE3) Chemically competent E. coli (44-0049; ThermoFisher, Waltham, MA). Ampicillin resistant colonies appeared on LB plates containing Ampicillin (A-301-100; Gold Biotechnology, St. Louis, MO) after overnight incubation at 37 °C.

A single colony was used to inoculate 10 ml of LB media with ampicillin (100 µg/ml) and grown with shaking, overnight at 30 °C. The pre-cultured bacteria were transferred to a 2800 ml flask containing 1000 ml LB Media with Ampicillin (500 µg/ml) and incubated at 37 °C for ~3 hours. Once the OD<sub>600</sub> reached 0.6-0.8, 0.25 mM isopropyl-beta-D-thiogalactopyranoside (IPTG; 12481C50; Gold Biotechnology, St. Louis, MO) was used to induce expression of GST-Δ40APE-1<sup>wt</sup> at 25 °C for 8 hours. The culture was then centrifuged at 5,000 rpm using ThermoScientific™ Sorvall LYNX 4000 Superspeed Centrifuge for 15 minutes, and pellets were harvested and resuspended in cold 1x phosphate-buffered saline (PBS, pH 7.4; 70011-044, Life technologies corporation, NY). The expression of GST-Δ40APE-1<sup>wt</sup>fusion protein was confirmed by 12% SDS-polyacrylamide gels. The resuspended pellets were lysed on ice via sonication at 70% amplitude in 1X PBS with 1 second on and 1 second off for 5 minutes. The sample was centrifuged at 17,000
rpm using ThermoScientific™ Sorvall Lynx 18,000 Superspeed Centrifuge for 45 minutes, and the supernatant which contained GST-Δ40APE-1^wt, was collected.

In order to purify the glutathione-S-transferase (GST) - Δ40APE-1^wt fusion protein, Glutathione Agarose Resin (G-250-50; Gold Biotechnology, St. Louis, MO) was added to the supernatant and mixed on a roller at 4 °C for 4 hours. GS4B beads provide a reversible ligand binding with GST-tagged protein, which leads to a mild purification process without any effect neither on protein structure nor its function. The supernatant and bead slurry were then added to gravity flow columns, and the unspecifically-bound proteins from the resin were washed thoroughly with 20 times of beads volume of cold 1X PBS. The GST-Δ40APE-1^wt protein was eluted from the column using 25 mM Reduced Glutathione pH 8.0 (GSH; G-155-100; Gold Biotechnology, St. Louis, MO). Buffer change of the eluates to 1X PBS was achieved using 10 kDa Vivaspin Ultrafiltration centrifugal tubes.

PreScission Protease is a fusion protein of glutathione S-transferase (GST). The protease recognizes explicitly the subset of sequences, which include the core amino acid sequence Leu-Phe-Gln/Gly-Pro cleaving between the Gln and Gly residues. This sequence is present between the GST moiety and the cloned Δ40APE-1^wt. 40 μl of PreScission Protease was added to the sample and incubated overnight at 4 °C. The cleavage of GST-Δ40APE-1^wt protein was confirmed by SDS-PAGE.

The cleaved protein sample was purified using a size exclusion HiLoad™ 16/600 SuperdexTM 75 pg (28-9893-33; GE Healthcare Bio-Science; Sweden) column equilibrated with Buffer A (20 mM Tris/HCl, pH 8.00, 150 mM NaCl, Degassed/ filtered). Then, the column, and run in buffer A with a flow rate of 1mL/min with the total run at 120 minutes. The absorbance at 280 nm was measured,
and fractions eluted at 40 minutes to 55 minutes (dimeric Δ40APE-1wt), 60 minutes to 72 minutes (monomeric Δ40APE-1wt), and 108 minutes to 120 minutes (mainly GST) were collected. The fractions were analyzed by 12% SDS-PAGE. A modified Laemmli sample buffer without 2-Mercaptoethanol (β-Mercaptoethanol/ BME) was used to avoid disruption of Δ40APE-1wt dimers. The Δ40APE-1wt protein was concentrated in 1X PBS buffer using 10 kDa Vivaspin Ultrafiltration centrifugal tubes at 21,130 rcf and 4 °C. Bradford assay was used to quantify the protein content and ~7 mM concentrated Δ40APE-1wt protein stored at -80 °C.

2.1.2 Δ40APE-1wt crystallization and X-ray crystallography

~7 mM Δ40APE-1wt protein was used to set up crystallization trails, in a 1:1 ratio with reservoir solution. Hampton Research and Rigaku Reagents crystallization kits were used. Monoclinic protein crystals were grown within in 3 days in sitting drops containing 0.2 M calcium chloride dihydrate, and 20% (w/v) Polyethylene glycol (PEG) 3,350 by vapor diffusion at 20 °C. Diffraction data were collected from single crystals, after a brief transfer to a suitable cryoprotectant (20% glycerol with the reservoir solution) using the X-ray diffraction system (D8 Venture Diffraction System, Bruker AXS Inc.) at our home institute and processed using PROTEUM2 (Bruker AXS Inc.). The model was then refined by the Refmac5. Initial phases were determined by Dr. Rahighi et al. by molecular replacement (MR) in the Molrep (CCP4), using the human APE-1 structure (PDB: 5CFG). The Δ40APE-1wt was modeled in a pocket at the Mg²⁺ channel interface based on strong electron density followed by successful refinement of the coordinates. Structure graphics and amino acid sequence alignment were prepared using PyMOL (2.1.0 version; TM; Schrödinger, LLC).
2.1.3 Δ40APE-1\(^{\text{wt}}\) protein crystallization under oxidative condition

The N-terminal truncated protein lacking the first 40 amino acid residues, Δ40APE-1\(^{\text{wt}}\) was purified, oxidized with 200 \(\mu\)M \(\text{H}_2\text{O}_2\), 10 \(\mu\)M Fe\(^{2+}\), and set up for crystallization trails as described above. Monoclinic protein crystals were grown in a sitting drop containing 100 mM MES/ sodium hydroxide pH 6.0, 20% W/V PEG 8,000, 200 mM calcium acetate by vapor diffusion at 20 °C. Monoclinic crystals grew within a week. Diffraction data were collected from single crystals, after a brief transfer to a suitable cryoprotectant (25% glycerol with the reservoir solution), on the X-ray diffraction system (D8 Venture Diffraction System, Bruker AXS Inc.) at our home institute and processed using PROTEUM2 (Bruker AXS Inc.). Initial phases were determined by molecular replacement (MR) using PHASER from the CCP4 by Young-Woo Nam et al. Our previously determined Δ40APE-1\(^{\text{wt}}\) structure was used as a starting model to phase diffraction data. Prior to modeling the rigid body refinement, solvent molecules were removed, and the crystallographic model was constructed through iterative rounds of manual model-building using Coot3 and further refinement by PHENIX60. The Δ40APE-1\(^{\text{wt}}\) was modeled in a pocket at the Mg\(^{2+}\) channel interface based on strong electron density in difference Fourier maps and followed by successful refinement of the coordinates. The crystallographic statistics for data collection and model refinement are summarized in Supplementary Table 1. Structure graphics, and amino acid sequence alignment were prepared using PyMOL (2.1.0 version; TM; Schrödinger, LLC).

2.1.4 Structural study of Δ40APE-1\(^{\text{wt}}\) after co-incubation with APE/Ref-1 inhibitor R21-OH

To determine the binding characteristics of Δ40APE-1\(^{\text{wt}}\), co-crystallization with our lead APE/Ref-1 inhibitor, R21-OH was performed. The Δ40APE-1\(^{\text{wt}}\) protein was expressed and purified as previously described. Approximately 5.7 mM concentrated purified protein was subjected for co-
incubation with our potent APE/Ref-1 inhibitor, R21-OH. The protein complex was mixed with R21-OH (50 mM) and was set up for crystallization in a 1:1 ratio with reservoir solution. Protein crystals of the protein complex were grown in sitting drops by vapor diffusion at 20 °C. Monoclinic protein crystals were grown within a week in sitting drops containing 0.2 M sodium chloride, 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.5, 20% (w/v) PEG 3,350 by vapor diffusion at 20 °C.

Diffraction data were collected from single crystals, after a brief transfer to a suitable cryoprotectant (20% glycerol with the reservoir solution) using the X-ray diffraction system (D8 Venture Diffraction System, Bruker AXS Inc.) at our home institute and processed using PROTEUM2 (Bruker AXS Inc.). The model was then refined by the Refmac5. Initial phases were determined by Dr. Rahighi et al. by molecular replacement (MR) in the Molrep (CCP4), using the human APE-1 structure (PDB: 5CFG). The Δ40APE-1wt was modeled in a pocket at the Mg2+ channel interface based on strong electron density followed by successful refinement of the coordinates. Structure graphics and amino acid sequence alignment were prepared using PyMOL (2.1.0 version; TM; Schrödinger, LLC).

2.1.5 Crystal soaking analysis of Δ40APE-1wt with APE/Ref-1 inhibitor R21-OH

Monoclinic protein crystals were grown in 0.2 M sodium chloride, 0.1 M HEPES pH 7.5, 20% (w/v) PEG 3,350, within 3 days at 20 °C. For “soaking” experiments, preformed crystals of the Δ40APE-1wt protein were incubated with our potent inhibitor at different concentrations of 10, 20, and 50 mM.
2.1.6 Detection of Δ40APE-1\textsuperscript{wt} dimer formation using Western Blot

The Δ40APE-1\textsuperscript{wt} protein was expressed and purified as previously described. Approximately 0.3 μg/ml Δ40APE-1\textsuperscript{wt} was incubated with 10mM TCEP (77720; Thermo Scientific, Rockford, IL) overnight. After washing out the reducing reagent, 5, 10, 20, and 50 of R21-OH and 1% DMSO (20688; Thermo Scientific, Rockford, IL) as a positive control was incubated with the reduced Δ40APE-1\textsuperscript{wt} overnight. Samples were then mixed with 5X denaturing loading buffer containing 10% SDS, loaded and separated on SDS-polyacrylamide gels. Overnight transfer was performed on an Immobilon-PSQ polyvinylidene difluoride (PVDF) membranes (ISEQ00010; Merck KGaA, Darmstadt, Germany).

To determine whether R21-OH facilitates dimerization of Δ40APE-1\textsuperscript{wt}, we further incubated Δ40APE-1\textsuperscript{wt} with 10 mM Tris (2-carboxyethyl) phosphine (TCEP; 77720; Thermo Scientific, Rockford, IL), at different time points to determine whether R21-OH facilitates dimerization of Δ40APE-1\textsuperscript{wt}. Then the reducing reagent is washed out and equal amount of Δ40APE-1\textsuperscript{wt} protein was incubated for another 24 hours with different concentrations of R21-OH and the negative control was treated with the same amounts of DMSO solution. Samples were then filtered, loaded and separated on SDS-polyacrylamide gels using 5X native dye containing 10% Sodium dodecyl sulfate (SDS), then transferred to Immobilon-PSQ polyvinylidene difluoride (PVDF) membranes (ISEQ00010; Merck KGaA, Darmstadt, Germany). Western blots were blocked using 10% non-fat milk, and then the membranes were incubated with the primary rabbit monoclonal anti-human Ape1 (1:10,000, #4128; Cell Signaling Technology, Danvers, MA) antibody overnight at 4 °C followed by secondary anti-rabbit antibody (1:2,000 Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature. Blots were washed extensively with TBS-T after each antibody incubation. Labeled bands were detected using SuperSignal horseradish peroxidase.
chemiluminescence reagents (1859674; 1859675; Thermo Fisher Scientific, Waltham, MA) and the bands were analyzed using the Bio-Rad ChemiDoc XRS+ System and normalized to ∆40APE-1wt levels.

2.1.7 Overexpression of ∆40APE-1C138A mutant protein

The single-cysteine mutant APE-1 proteins tagged at the N-terminus with GST plasmid construct were sent for site-directed mutagenesis of cysteine 138 to alanine (GeneScript, Piscataway, NJ), the protein was expressed as described previously. In a brief, a single-cysteine mutant protein, was cloned into the pGEX-6P1 vector. The ∆40APE-1C138A construct was then transfected into the one shot® BL21 Star™ (DE3) Chemically competent E.Coli (44-0049; ThermoFisher, Waltham, MA) by heat shock at 42 °C for 45 seconds. Selected colonies were inoculated and grown overnight at 30 °C in 50 mL Luria broth (LB) medium containing 100 μg/mL ampicillin (A-301-100; Gold Biotechnology, St. Louis, MO). 5 ml of pre-cultured bacteria were transferred to a 2800 ml flask containing 1000 ml LB Media with ampicillin (500 μg/ml) and incubated at 37 °C for 3 hours. Once the OD$_{600}$ reached 0.6-0.8, induction was performed with 0.25 mM isopropyl-beta-D-thiogalactopyranoside (IPTG; 12481C50; Gold Biotechnology, St. Louis, MO) at 25 °C for 8 hours. The culture was then centrifuged at 5,000 rpm using ThermoScientific™ Sorvall LYNX 4,000 Superspeed Centrifuge for 15 minutes, and pellets were collected and resuspended in cold Phosphate Buffered Saline (1X PBS, pH 7.4). The expression of fusion protein before and after induction is confirmed by 12% SDS-polyacrylamide gels. The resuspended pellets were lysed on ice via sonication at 70% amplitude in 1X PBS with 1 second on and 1 second off for 5 minutes. The sample was centrifuged at 17,000 rpm using ThermoScientific™ Sorvall LYNX 4000 Superspeed Centrifuge for 45 minutes, and the supernatant which contained the protein of interest, was collected.
2.1.8 Pull down assay using GST-Δ40APE-1\textsuperscript{wt} and GST-Δ40APE-1\textsuperscript{C138A} protein

Nuclear extraction of melanoma A375 cells were treated with 100 μM H\textsubscript{2}O\textsubscript{2} for 72 hours in serum-free media and were collected via centrifugation and resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.150 mM MgCl\textsubscript{2}, 0.5 mM DTT, 0.2 mM PMSF) with 1% protease inhibitor cocktail (PIC) and allowed to swell for 10 minutes. 10% NP-40 was added and vortexed to lyse the cells. The sample was spun down at maximum speed for 30 seconds to collect the cytosolic fraction. The pelleted nuclei were resuspended and incubated in buffer C (20 mM HEPES, 20% glycerol, 0.42 M NaCl, 0.15 mM MgCl\textsubscript{2}, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) with 1% PIC then placed in the centrifuge at maximum speed for 30 minutes to isolate the nuclear proteins. Buffer D (20 mM HEPES, 20% glycerol, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) was then added to dilute the nuclear samples. Protein concentration was determined by Bradford assay. GST-Δ40APE-1\textsuperscript{wt} and GST-Δ40APE-1\textsuperscript{C138A} purified protein (100 μg) were oxidized separately overnight by incubation with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, 200 μM) and Fe\textsuperscript{2+} (5 μM or 10 μM) respectively. After washing out the hydrogen peroxide and Fe\textsuperscript{2+}, samples were incubated overnight with equal amounts of nuclear extractions on Glutathione Agarose Resin (G-250-50; Gold Biotechnology, St. Louis, MO). Elution was performed using 25mM Reduced Glutathione pH 8.0 (GSH; G-155-100; Gold Biotechnology, St. Louis, MO), followed by buffer change to PBS. Samples were then loaded and separated on SDS-polyacrylamide gels, then transferred to Immobilon-PSQ polyvinylidene difluoride (PVDF) membranes (ISEQ00010; Merck KGaA, Darmstadt, Germany) for Western blotting analysis.

Membranes blocked by 10% non-fat milk, were further incubated with the primary antibodies for 1 hour at room temperature or overnight at 4 °C, followed by secondary antibody incubation for 1
hour at room temperature. Membranes were washed extensively with TBS-T (Tris-buffered saline containing 0.1% Tween 20) after each antibody incubation. The specific bands were detected using SuperSignal horseradish peroxidase chemiluminescence reagents (1859674, 1859675; Thermo Fisher Scientific, Waltham, MA) and images were captured and analyzed using the Bio-Rad ChemiDoc XRS+ System.

2.1.9 Effect of cysteine 138 in APE/Ref-1 protein dimerization Δ40APE-1C138A

To further study the role of Cysteine 138 in the dimerization of APE/Ref-1 protein, we generated a single Cys138 mutant Δ40APE-1C138A, which cysteine was replaced by redox-inactive alanine using site-directed mutagenesis as directed by the manufacturer (GeneScript, Piscataway, NJ). The Δ40APE-1C138A construct was then transfected into the one shot® BL21 Star™ (λDE3) Chemically competent E.Coli (44-0049; ThermoFisher, Waltham, MA), and subjected for expression and purification as described previously in section 2.1.7.

To determine the effect of Cys138A on protein dimerization under oxidative condition, the purified Δ40APE-1C138A protein was first incubated with 200 µM H₂O₂ overnight. After washing out H₂O₂ using EMD Millipore™ Amicon™ Ultra-0.5 Centrifugal Filter Units (UFC501024; ThermoFisher, Waltham, MA), equal amount of Δ40APE-1wt protein samples in PBS were incubated with different concentrations R21-OH for 24 hours. The sample incubated with the same amounts of DMSO solution was defined as control. After incubation, samples were then washed, filtered, and subjected for SDS-polyacrylamide gels using 5x native dye-containing 10% sodium dodecyl sulfate (SDS). Samples were then transferred to Immobilon-PSQ polyvinylidene difluoride (PVDF) membranes (ISEQ00010; Merck KGaA, Darmstadt, Germany) for blotting and imaging using specific antibodies as described in 2.1.6.
2.2 Cell lines, chemicals and reagents

The human melanoma cell line A375 was obtained from American Type Culture Collection (ATCC; Manassas, VA), and was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; #11995073; Gibco, Waltham, MA) with 10% fetal bovine serum (FBS; #26140079; Gibco, Waltham, MA), or Tumor Specialized Media with 2% FBS (WM3211, 1205Lu).

Novel APE/Ref-1 inhibitor, R21-OH, designed and synthesized by Yang et al. and Parang et al. research groups respectively, were dissolved in 100 mM DMSO (20688; Thermo Scientific, Rockford, IL) for stock concentration. Further, serial dilutions of R21-OH were used for experimental use.

2.2.1 Antibodies

Mouse monoclonal anti-human STAT3, Nrf-2 (A-10) (sc-293151; sc365949; Santa Cruz Biotechnology, Inc. Dallas, TX) and rabbit monoclonal anti-human Ape1 (#4128; Cell Signaling Technology, Danvers, MA) antibodies were used as primary antibodies; horseradish peroxidase-labeled anti-mouse and anti-rabbit (1:5,000; 1:2,000 Cell Signaling Technology, Danvers, MA) was used as the secondary antibodies.

2.3 Surface Plasmid Resonance (SPR)

Purified recombinant GST-Δ40APE-1wt protein (50 μg/ml) was conjugated on GST sensor chip (Nicoya Lifesciences Open SPR) using GST ligand wizard immobilization (Instrument standard procedures) followed by 50 μg/ml GST blocking. Serial dilution of R21-OH analyte (0.25, 0.5, and 1 mM in running buffer containing 1.02% PBS with 0.5% Dimethylformamide (DMF) and 0.5% Methanol (MeOH) was injected at a flow rate of 20 μl/min sequentially into the chamber in
running buffer (1.02% PBS, 0.5% DMF and 0.5% MeOH). The interaction of GST fusion protein with R21-OH was detected by OpenSPR™ (Nicoya Lifesciences) at 20 °C and allowed to associate and dissociate for 120 seconds and 480 seconds, respectively. The surface chip was regenerated with 25 mM GSH solution in 0.5 M Tris-HCl pH 8.0 at flow rate of 150 μl/min. The close curve fitting to the sensorgrams was calculated by global fitting curves (1:1 Langmuir binding model). The data was retrieved and analyzed with TraceDraw software.

Using the same techniques and running buffer, the purified recombinant mutated GST-Δ40APE-1C138A (50 μg/ml) protein was conjugated on GST sensor chip of OpenSPR™ (Nicoya Lifesciences) and interactions between the ligand and the analyte was detected. The close curve fitting to the sensorgrams was calculated by global fitting curves (1:1 Langmuir binding model). The data was retrieved and analyzed with TraceDraw software.
Chapter 3

3.1 Results

3.1.1 Δ40APE-1\textsuperscript{wt} protein expression

First, N-terminally truncated APE/Ref-1 protein lacking the first 40 amino acid residues (Δ40APE-1\textsuperscript{wt}) was cloned into the pGEX-6P1 vector to express the GST-tagged fusion protein. As shown in Figure 3.1, we have successfully expressed GST-Δ40APE-1\textsuperscript{wt} protein after induction with 0.25 mM IPTG. The targeted mass of 56.5 kDa is confirmed using 12% SDS-polyacrylamide gel. The GST tag was then subjected for cleavage using PreScission Protease enzyme (<0.1 EU/µg) at 4 °C. The 12% SDS-polyacrylamide gel results of the affinity chromatography shown in Figure 3.2, confirms partial digestion of the GST-tag from Δ40APE-1\textsuperscript{wt} with the target mass of 31.3 kDa after 24 hours incubation.

After GST tag cleavage, size exclusion chromatography was performed to separate the molecules based on their molecular weight. Samples were run through the HiLoad\textsuperscript{TM} 16/600 Superdex\textsuperscript{TM} 75 pg (28-9893-33; GE Healthcare Bio-Science; Sweden) column, and the two fraction pecks shown in Figure 3.3 A, were collected in running buffer, 20 mM Tris/Cl, pH 8.00, 150 mM NaCl.

The fraction collected from A32-A37 (from 60 minutes to 72 minutes) shown in Figure 3.3 A, is confirming the band for Δ40APE-1\textsuperscript{wt} protein by 12% SDS-polyacrylamide gel shown in Figure 3.3 B, and by western blot using rabbit monoclonal anti-human Ape1 primary antibodies respectively shown in Figure 3.3 C.
Figure 3.1 GST-Δ40APE-1<sup>wt</sup> expression after induction with 0.25 mM IPTG with 12% SDS-polyacrylamide gel. Considering the molecular weight of 31.3 kDa for truncated Δ40APE-1<sup>wt</sup> with 25 kDa GST-tag, the 56.5 kDa band confirmed targeted mass for GST-Δ40APE-1<sup>wt</sup>. 

<table>
<thead>
<tr>
<th>kDa</th>
<th>M</th>
<th>−</th>
<th>+</th>
</tr>
</thead>
<tbody>
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<td>57</td>
<td></td>
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<td>32</td>
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<tr>
<td>25</td>
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</tr>
</tbody>
</table>
Figure 3.2 Partial digestion of Δ40APE-1<sup>wt</sup> protein from Gst-tag (25 kDa) after incubation with PreScission Protease enzyme was confirmed by 12% SDS-polyacrylamide gels. Targeted mass of 31.3 kDa was confirmed for cleaved Δ40APE-1<sup>wt</sup> protein.
Figure 3.3 A) Size exclusion chromatography of the cleaved samples. Samples were injected to the HiLoadTM 16/600 SuperdexTM 75 pg (28-9893-33; GE Healthcare Bio-Science; Sweden) column in running buffer containing 20 mM Tris/HCl pH 8.0, 150 mM NaCl. The Δ40APE-1\textsuperscript{wt} peak was confirmed by 12% SDS-polyacrylamide gel, and Western blot using APE1 antibody. B) Confirmation of the Δ40APE-1\textsuperscript{wt} peak at A30-37 by 12% SDS-polyacrylamide gel. The targeted molecular weight of 31.3 kDa confirmed the band for Δ40APE-1\textsuperscript{wt}. C) Confirmation of the Δ40APE-1\textsuperscript{wt} protein fraction peak at A30-37 by western blot using APE1 antibody.
3.1.2 Crystal structure of Δ40APE-1\textsuperscript{wt}

7 mM Δ40APE-1\textsuperscript{wt} protein was subjected for crystallization. We have successfully obtained Δ40APE-1\textsuperscript{wt} crystals grown in sitting drops by vapor diffusion at 20 °C. Figure 3.4, shows an overview of some of the obtained Δ40APE-1\textsuperscript{wt} crystals in the specified resveratrol solution with their obtained diffraction pattern. The Δ40APE-1\textsuperscript{wt} crystal grown in sitting drop containing 0.2 M calcium chloride dihydrate, and 20% (w/v) polyethylene glycol (PEG) 3,350 shown in Figure 3.5 A), was subjected for screening using in house D8 Venture Diffraction System, Bruker AXS Inc. Figure 3.5 B), shows diffraction pattern of the subjected crystal with the specified unit cells that was collected with resolution up to 1.57Å. The data was processed using PROTEUM2 (Bruker AXS Inc.), and unit cells were defined as monoclinic with space group C2. The initial phases were determined by Dr. Rahighi et al. by molecular replacement (MR) in the Molrep (CCP4), using the already available human APE-1 structure (PDB: 5CFG). The Δ40APE-1\textsuperscript{wt} was modeled in a pocket at the Mg\textsuperscript{2+} channel interface based on strong electron density followed by successful refinement by the Refmac5. Structure graphics and amino acid sequence alignment were prepared using PyMOL (2.1.0 version; TM; Schrödinger, LLC). As can be seen in Figure 3.6, our solved Δ40APE-1\textsuperscript{wt} crystal structure highlighted in blue is overlapping with the already available human APE-1 structure (PDB: 5CFG) highlighted in green.
<table>
<thead>
<tr>
<th>Δ40APE-1&lt;sup&gt;st&lt;/sup&gt; Crystals</th>
<th>Resveratrol solution</th>
<th>Diffraction pattern</th>
<th>Crystal specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salt:</strong></td>
<td>N/A</td>
<td></td>
<td><strong>Resolution:</strong> 1.9Å</td>
</tr>
<tr>
<td><strong>Buffer:</strong></td>
<td>200 mM potassium nitrate</td>
<td></td>
<td><strong>Unit Cells:</strong></td>
</tr>
<tr>
<td><strong>Precipitant:</strong></td>
<td>20% (w/v) PEG 3350</td>
<td></td>
<td>a: 86.25Å  α: 90.00°</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b: 45.69Å  β: 105.24°</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c: 78.52Å  γ: 90.00°</td>
</tr>
<tr>
<td><strong>Space Group:</strong></td>
<td>Monoclinic C</td>
<td></td>
<td><strong>Salt:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
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<td><strong>Buffer:</strong></td>
<td>200mM calcium chloride</td>
<td></td>
<td><strong>Resolution:</strong> 2.22Å</td>
</tr>
<tr>
<td><strong>Precipitant:</strong></td>
<td>20% W/v PEG 3,350</td>
<td></td>
<td><strong>Unit Cells:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>a: 77.09Å  α: 90.00°</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b: 44.85Å  β: 92.20°</td>
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<td></td>
<td>c: 77.51Å  γ: 90.00°</td>
</tr>
<tr>
<td><strong>Space Group:</strong></td>
<td>Monoclinic C</td>
<td></td>
<td><strong>Salt:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.2M calcium acetate hydrate</td>
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<tr>
<td><strong>Buffer:</strong></td>
<td>20% w/v polyethylene glycol 3350 pH 7.5</td>
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<td><strong>Resolution:</strong> 1.74Å</td>
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<tr>
<td><strong>Precipitant:</strong></td>
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<td></td>
<td><strong>Unit Cells:</strong></td>
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<td></td>
<td></td>
<td></td>
<td>a: 75.71Å  α: 90.0°</td>
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<td></td>
<td></td>
<td></td>
<td>b: 44.75Å  β: 91.90°</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>c: 76.73Å  γ: 90.0°</td>
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<tr>
<td><strong>Space Group:</strong></td>
<td>Monoclinic C</td>
<td></td>
<td></td>
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</tbody>
</table>

Figure 3.4 Overview of Δ40APE-1<sup>st</sup> crystals. The reservoir solution for each obtained crystal along with diffraction specification is mentioned in the Figure.
Figure 3.5 A) Δ40APE-1wt crystal grown by vapor diffusion at 20 °C in sitting drop containing 0.2 M calcium chloride dihydrate, and 20% (w/v) polyethylene glycol (PEG) 3,350 was subjected for screening. B) Diffraction pattern obtained from crystal grown in sitting drop containing 0.2 M calcium chloride dihydrate, and 20% (w/v) polyethylene glycol (PEG) 3,350 was collected in house using D8 Venture Diffraction System, Bruker AXS Inc and the data was processed using PROTEUM2 (Bruker AXS Inc.). The data was collected with resolution up to 1.57Å and the unit cells were defined as monoclinic C2.
Figure 3.6 Δ40APE-1<sup>wt</sup> monomer structure. As can be seen in the figure, our solved structure of Δ40APE-1<sup>wt</sup> protein highlighted in blue is overlapping with the reported APE/Ref-1 structure in protein data bank (PDB: 5CFG), highlighted in green.
3.1.3 Co-incubation of Δ40APE-1\textsuperscript{wt} with novel APE/Ref-1 inhibitor, R21-OH

We then conducted co-crystallization of Δ40APE-1\textsuperscript{wt} with our novel design APE/Ref-1 inhibitor R21-OH (50 µM). Figure 3.7 shows some of the obtained co-crystals with their specified resveratrol solution and their diffraction pattern specifications. The crystal grown in sitting drop containing 0.2 M calcium chloride, 0.1 M HEPES pH 7.5 and 20% (w/v) polyethylene glycol (PEG) 3,350, shown in Figure 3.8 A), was subjected for screening using in house D8 Venture Diffraction System, Bruker AXS Inc. As can be seen in Figure 3.8 B), diffraction pattern of the subjected crystal was collected with resolution of 2.48Å. The data was processed using PROTEUM2 (Bruker AXS Inc.), and unit cells were defined as monoclinic with space group C2.

The initial phases were determined by Dr. Rahighi et al. by molecular replacement (MR) in the Molrep (CCP4), using the already available human APE-1 structure (PDB: 5CFG). The Δ40APE-1\textsuperscript{wt} was modeled in a pocket at the Mg\textsuperscript{2+} channel interface based on strong electron density followed by successful refinement by the Refmac5. Structure graphics and amino acid sequence alignment were prepared using PyMOL (2.1.0 version; TM; Schrödinger, LLC).
Figure 3.7 Overview of co-incubation of Δ40APE-1wt with 50 µM R21-OH. The reservoir solution for each obtained co-crystal along with diffraction specification is mentioned in the figure.
Figure 3.8 A) Co-incubated Δ40APE-1wt protein crystal in 50 μM R21-OH, obtained from vapor diffusion at 20 °C in sitting drop containing 0.2 M calcium chloride, 0.1 M HEPES pH 7.5 and 20% (w/v) polyethylene glycol (PEG) 3,350 was subjected for screening. B) Diffraction pattern was collected in house using D8 Venture Diffraction System, Bruker AXS Inc and the data was processed using PROTEUM2 (Bruker AXS Inc.). The data was collected with resolution of 2.48Å and the unit cells were defined as monoclinic C2.
3.1.4 Novel APE/Ref-1 inhibitor, R21-OH, facilitates dimerization of \( \Delta 40 \text{APE-1}^{\text{wt}} \) protein at Cys 138 residue

Figure 3.9 shows our solved structure of \( \Delta 40 \text{APE-1}^{\text{wt}} \) with R21-OH (50 µM). The model was refined by the Refmac5, and the molecular replacement (MR) was performed by Molrep (CCP4) software, using already available human APE-1 structure (PDB: 5CFG). The \( \Delta 40 \text{APE-1}^{\text{wt}} \) was modeled in a pocket at the \( \text{Mg}^{2+} \) channel interface based on strong electron density in difference Fourier maps and followed by successful refinement of the coordinates. Our solved structure shown in Figure 3.9 failed to identify any binding of R21-OH with \( \Delta 40 \text{APE-1}^{\text{wt}} \) protein. However, the formation of the disulfide bond at cysteine 138 residues between the two \( \Delta 40 \text{APE-1}^{\text{wt}} \) monomers was observed.
Our solved co-incubated structure failed to identify any binding of R21-OH with Δ40APE-1wt. However, the formation of disulfide bond is evident at Cys138.
3.1.5 Crystal structure of APE/Ref-1 dimer under oxidative stress

~5 mM Δ40APE-1wt protein was subjected for crystallization under oxidative condition with H$_2$O$_2$ (200 µM)/Fe$^{2+}$ (10 µM) for crystallization. Successfully obtained crystals in oxidative condition with their diffraction specifications are shown in Figure 3.10. One of the obtained oxidized Δ40APE-1wt crystals grown in sitting drop containing 100 mM MES/NaOH pH 6.0, 0.1 M HEPES pH 7.5, 20% w/v PEG 8,000, and 200 mM calcium acetate (Figure 3.11 A) was subjected for screening using in house D8 Venture Diffraction System, Bruker AXS Inc. Figure 3.11 B) shows the obtained diffraction pattern with resolution of 2.48Å. The data collection was processed using PROTEUM2 (Bruker AXS Inc.), and unit cells were defined as monoclinic with space group C2.
Figure 3.10 Overview of Δ40APE-1<sup>wt</sup> crystallization in oxidized condition. The reservoir solution for each obtained crystal along with diffraction pattern and specifications are mentioned in the Figure.
Figure 3.11 A) Δ40APE-1wt crystals formed under oxidative condition in sitting drop containing 100 mM MES/ sodium hydroxide pH 6.0, 0.1 M HEPES pH 7.5, and 20% (w/v) polyethylene glycol (PEG) 8,000 with 200 mM calcium acetate. B) X-ray diffraction data were collected using D8 Venture Diffraction System (Bruker AXS Inc.) and further processed using PROTEUM2 program (Bruker AXS Inc.). The crystal used for data collection had a resolution of 2.3Å and the unit cells were defined as monoclinic C2.
3.1.6 Dimerization of Δ40APE-1wt protein under oxidative stress

The obtained crystal under oxidative stress was subjected for data collection. The initial phases were determined by Young-Woo Nam et al. by molecular replacement (MR) in the Molrep (CCP4), using the already available human APE-1 structure (PDB: 5CFG). The data was processed using PROTEUM2 (Bruker AXS Inc.), and the unit cells were defined as monoclinic with space group C2. Prior to modeling the rigid body refinement, solvent molecules were removed, and the crystallographic model was constructed through iterative rounds of manual model-building using Coot3 and further refinement by PHENIX60. The Δ40APE-1wt was modeled in a pocket at the Fe2+ channel interface based on strong electron density in difference Fourier maps and followed by successful refinement of the coordinates. Structure graphics and amino acid sequence alignment shown in Figure 3.12 were prepared using PyMOL (2.1.0 version; TM; Schrödinger, LLC). The solved structure determined formation of disulfide bond between the Cys138 residues of two APE/Ref-1 molecules (Figure 3.12). To the best of our knowledge, this is the first reported dimer structure of APE/Ref-1 formed under oxidative condition.
Figure 3.12. Structure of ∆40APE-1wt protein under oxidative stress (200 µM H₂O₂/10 µM Fe²⁺). Formation of disulfide bond between the Cys138 residues of two APE/Ref-1 molecules is evident in our solved structure. To the best of our knowledge, this is the first reported dimer structure of APE/Ref-1 formed under oxidative condition.
Table 1. Data collection and refinement of oxidized Δ40APE-1wt with 200 µM H₂O₂ +10 µM Fe²⁺

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Oxidized Δ40APE-1wt (200 µM H₂O₂ +10 µM Fe²⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>1.3134</td>
</tr>
<tr>
<td>Space group</td>
<td>C 1 2 1</td>
</tr>
<tr>
<td>Unit cells (Å)</td>
<td>A=76.52, b=44.87, c=77.44 Å</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>25.80.78 -2.3 (2.38-2.3)</td>
</tr>
<tr>
<td>Total reflection</td>
<td>41,170 (2823)</td>
</tr>
<tr>
<td>Unique reflection</td>
<td>11,765 (1096)</td>
</tr>
<tr>
<td>Completeness</td>
<td>98.5 (92.4)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.5 (2.6)</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>9.8 (3.2)</td>
</tr>
<tr>
<td>R_merge</td>
<td>0.101 (0.342)</td>
</tr>
<tr>
<td>CC1/2</td>
<td>0.995 (0.926)</td>
</tr>
</tbody>
</table>

Refinement

| Resolution | 25.8 - 2.3 |
| No.of reflection | 11883 |
| R factor/ R_free (%) | 0.2027/ 0.2896 |
| No. of atoms | 2322 |
| No. of solvents | 128 |

RMSD from ideal values

| Bond lengths (Å) | 0.009 |
| Bond angels (°) | 1.34 |

Ramachandran plot (%)

| Favored | 95.60 |
| Allowed | 4.4  |
| Outlier | 0    |

---

1 Calculated using XDS.
2 Values in parentheses are for the highest resolution shell
3.1.7 The formation of Δ40APE-1<sup>wt</sup> dimer detected by western blot analysis

Figure 3.13 To determine whether R21-OH facilitates dimerization of APE/Ref-1, we conducted western blot experiment by starting incubation of Δ40APE-1<sup>wt</sup> protein (0.5 mg) with 10 mM TCEP (reducing reagent) at room temperature for 24 hours, 48 hours and 72 hours respectively. Samples were collected for SDS-PAGE, followed by blotting with anti-APE/Ref-1 antibody. The bands were scanned and analyzed by Bio-Rad ChemiDoc XRS+ system. Figure 3.13 A) shows the western blot data of Δ40APE-1<sup>wt</sup> (0.5 mg) incubated with 10 mM TCEP. As shown in the results, after incubation for 72 hours, majority of Δ40APE-1<sup>wt</sup> is presented with monomer form and the conversion of dimer to predominant monomer state of Δ40APE-1<sup>wt</sup> protein is evident in reduced condition (exposed with 10 mM TCEP). Figure 3.13 B) shows the co-incubated of the post treated Δ40APE-1<sup>wt</sup> protein with reducing reagent, followed by incubation with different concentrations of R21-OH The bands were scanned and analyzed by Bio-Rad ChemiDoc XRS+ system and normalized to Δ40APE-1<sup>wt</sup> levels. The experiment was conducted at least three times and the dimer bands were normalized to Δ40APE-1<sup>wt</sup> levels. As can be seen the results shown in Figure 3.13 B). As shown in the figure, Δ40APE-1<sup>wt</sup> protein is converted to dimer state after co-incubation with R21-OH. Our results indicate that our potent inhibitor, R21-OH facilitates dimerization of Δ40APE-1<sup>wt</sup> in a dose dependent manner, which can be a novel APE/Ref-1 mechanism in anti-melanoma mechanism.
Figure 3.13 A) shows our Western Blot experiment of ∆40APE-1\textsuperscript{wt} co-incubated with TCEP (10 mM reducing reagent) and is detected by anti-APE/Ref-1 antibody. As shown in the results, after incubation with TCEP (10 mM) for 72 hours, majority of ∆40APE-1\textsuperscript{wt} is presented with monomer form and the conversion of dimer to predominant monomer state of ∆40APE-1\textsuperscript{wt} protein is evident in reduced condition (exposed with 10 mM TCEP). B) shows the results after incubation of the reduced ∆40APE-1\textsuperscript{wt} protein with different concentrations of R21-OH. The protein is converted to dimer state after co-incubation with R21-OH in a dose dependent manner. Our results indicate that our potent inhibitor, R21-OH facilitates dimerization of ∆40APE-1\textsuperscript{wt}, which might be a novel mechanism of its anti-melanoma activities.
3.1.8 ∆40APE-1C138A protein expression

In order to determine if the binding of ∆40APE-1\textsuperscript{wt} with Nrf-2 was redox-dependent or not, we constructed a redox-deficient APE/Ref-1 mutant with cysteine 138 replaced by alanine. The ∆40APE-1\textsuperscript{C138A} mutant protein was expressed and purified in exactly the same way as ∆40APE-1\textsuperscript{wt} as mentioned in 2.1.1. The GST-∆40APE-1\textsuperscript{C138A} protein was subjected for GST-tag cleavage using in house PreScission Protease enzyme (<0.1 EU/µg) at 4 °C. As shown in Figure 3.14 A) the partial digestion of GST-tag protein is confirmed by 12% SDS-polyacrylamide gel. After GST-tag cleavage, size exclusion chromatography is performed to separate the molecules based on their molecular weight. Samples were run through the HiLoad\textsuperscript{TM} 16/600 Superdex\textsuperscript{TM} 75 pg (28-9893-33; GE Healthcare Bio-Science; Sweden) column, in running buffer containing 20 mM Tris/HCl, pH 8.00, 150 mM NaCl. The subjected fractions peck at 72 minutes to 84 minutes shown in Figure 3.14 B) were collected and as can be seen in Figure 3.14 C), samples were analyzed by 12% SDS-polyacrylamide gel.
Figure 3.14  

A) Partial digestion of GST-Δ40APE-1C138A mutant protein by PreScission Protease enzyme (<0.1 EU/µg).  

B) Size exclusion chromatography of the cleaved GST-Δ40APE-1C138A mutant protein. Samples were injected to the HiLoadTM 16/600 SuperdexTM 75 pg column in running buffer containing 20 mM Tris/ HCl pH 8.0, 150 mM NaCl. The subjected fractions peck at 72 minutes to 84 minutes confirmed Δ40APE-1C138A protein by 12% SDS-polyacrylamide gel (C).  

C) The targeted molecular weight of 31.3 kDa confirmed the band for Δ40APE-1C138A.
3.1.9 Δ40APE-1C138A mutant protein failed to form dimerization under oxidative condition and co-incubation with R21-OH

We conducted the same experiment mentioned before 2.1.6 using Δ40APE-1C138A mutant protein. Figure 3.15 show our western blot experiment of Δ40APE-1C138A exposed with H₂O₂ (200 μM), and different concentrations of the novel APE/Ref-1 inhibitor, R21-OH at room temperature overnight. As can be seen in Figure 3.15, the distinct redox form of Δ40APE-1wt, was failed to observe any dimer formation of Δ40APE-1C138A mutated protein under oxidative condition (200 μM H₂O₂), and with co-incubation with R21-OH for 12 hours. Based on the results shown in Figure 3.15, the monomer remains the predominant form of Δ40APE-1C138A protein which demonstrates the critical role of Cys138 in the dimerization of Δ40APE-1wt. Further study is warranted to determine the role of Cys138 in the binding of Nrf-2 protein.

![Figure 3.15](image)

Figure 3.15 Δ40APE-1C138A mutant protein exposed with H₂O₂ (200 μM), and different concentrations of the novel APE/Ref-1 inhibitor, R21-OH at room temperature overnight. The distinct redox form of Δ40APE-1wt shown in Figure 3.13 was failed to observe any dimer formation of Δ40APE-1C138A mutated protein specially under oxidative condition (200 μM H₂O₂), and with co-incubation with R21-OH for 12 hours.
3.1.10 The binding of APE/Ref-1 with Nrf-2 was regulated in a redox-dependent manner

To investigate whether the signaling pathway from APE/Ref-1 to the nuclear transcription factors is regulated in a redox-dependent manner, we performed GST pulldown analysis with the GST-Δ40APE-1<sup>wt</sup> protein and the nuclear extracts of melanoma cells. Δ40APE-1<sup>wt</sup> expressed and purified as described before 2.1.1. Purified GST-Δ40APE-1<sup>wt</sup> protein was oxidized by H<sub>2</sub>O<sub>2</sub> (200 μM)/Fe<sup>2+</sup> (5 μM and 10 μM) overnight, followed by washing and centration spin to remove H<sub>2</sub>O<sub>2</sub>/Fe<sup>2+</sup>. 100 μg of GST-Δ40APE/Ref-1<sup>wt</sup> was immobilized on glutathione beads and incubated with nuclear extraction of melanoma cells at room temperature overnight [67]. Beads were then washed, pelleted, boiled in SDS sample buffer, and subjected to immunoblot analysis. As shown in Figure 3.16 A) As can be seen in the results, the pre-treated cells with H<sub>2</sub>O<sub>2</sub> induced binding of Δ40APE-1<sup>wt</sup> to nuclear transcription factors such as Nrf-2 and STAT3. While, the binding is significantly reduced in post-treatment with H<sub>2</sub>O<sub>2</sub>, suggesting that the oxidized APE/Ref-1 protein impaired its binding capacity to nuclear transcription factors.

We further conducted the same GST pull down analysis experiment using GST-Δ40APE-1<sup>C138A</sup> mutant protein. The GST-Δ40APE-1<sup>C138A</sup> mutant protein expressed and purified as described before 2.1.1. Purified GST-Δ40APE-1<sup>C138A</sup> mutant protein was oxidized by H<sub>2</sub>O<sub>2</sub> (200 μM)/Fe<sup>2+</sup> (5 μM and 10 μM) overnight, followed by washing and centration spin to remove H<sub>2</sub>O<sub>2</sub>/Fe<sup>2+</sup>. 100 μg of the protein was immobilized on glutathione beads and incubated with nuclear extraction of melanoma cells at room temperature overnight [67]. Beads were then washed, pelleted, boiled in SDS sample buffer, and subjected to immunoblot analysis. The bands were scanned and analyzed by Bio-Rad ChemiDoc XRS+ system and normalized to GST-Δ40APE-1<sup>C138A</sup> levels. As shown in the Figure 3.16 B) the mutated Δ40APE-1<sup>C138A</sup> protein significantly reduced binding of Δ40APE-
C138A to STAT3 compared to control, suggesting that the Cys138 is critical for the direct binding of APE/Ref-1 with STAT3.

Figure 3.16 A) Pull-down analysis results of the GST-Δ40APE-1wt protein with nuclear extraction of melanoma cells. The experiment was repeated at least three times and as can be seen in the results, the pre-treated cells with H2O2 induced binding of Δ40APE-1wt to Nrf-2 and STAT3. While, the binding is significantly reduced by oxidization of
Δ40APE-1<sup>wt</sup> with H<sub>2</sub>O<sub>2</sub>, which can be suggested that the redox status of APE/Ref-1 protein not only affects its dimerization but may also regulate its redox activity. B) Shows the same experiment using mutated Δ40APE-1<sup>wt</sup> protein. As shown in the results, after mutation of Cys138, the binding of Δ40APE-1<sup>C138A</sup> protein to the TFs such as STAT3 has been significantly reduced in pre-treated cells with H<sub>2</sub>O<sub>2</sub> compared to Δ40APE-1<sup>wt</sup> (results in A). This result may validate our hypothesis that the redox activity of Δ40APE-1<sup>wt</sup> is affected by its dimerization.
4 Discussion

Human cutaneous melanoma is among the most fatal and the most drug resistance of all malignancies in the United States in which the incidence rate continues to increase, making this disease a rising public health concern [2]. Invasive melanoma accounts for less than 2% of all skin cancer cases, but the vast majority of skin cancer deaths [68]. Despite powerful immunotherapy approaches for the treatment of cutaneous melanoma (CM), these inhibitors are mainly indicated for patients with advanced melanoma, leaving the world-wide melanoma epidemic and the incidence to rise [5, 69, 70]. APE/Ref-1, first recognized as a DNA endonuclease base excision repair (BER) enzyme that is found to plays an essential role in cancer cells survival from oxidative stress associated with radiation therapy and chemotherapy [71-73]. Abnormal expression of APE/Ref-1 in many human malignancies, including melanoma, has spurred efforts in the development of APE/Ref-1 novel redox inhibitors with redox regulation recognition mediated signaling.

To date, the development of the most successful, clinically available APE/Ref-1 inhibitor, APX3330 (NCT03375086), also known as E3330, has shown markedly decrease mediate oxidative stress compared with melanocytes [8]. E3330 exhibited therapeutic effects on tumor angiogenesis and growth with selective redox recognition without altering APE/Ref-1 endonuclease activity [74-77]. However, such inhibitory activity is mainly evident at higher concentrations of 50 µM [78]. Studies have indicated the redox signaling pathways of APE/Ref-1 via reduction of oxidized cysteine residues at certain nuclear transcription factors (TFs), converting them to a reduced state, resulting in the upregulation of tumor-promoting genes [8]. As such,
blocking APE/Ref-1 signaling pathway may inhibit broader downstream regulatory pathways, suggesting being more effective and efficient in melanoma suppression. To date, a limited number of compounds are proposed to exhibit APE/Ref-1 inhibitory pathways such as CRT0044876 (AP endonuclease site inhibitory; Koll et al., 2008; Madhusudan et al., 2005), and PNRI-299 (AP-1 transcription activation with little effect on NF-kB; Nguyen et al., 2003). However, biological activity analyses of such novel inhibitors demonstrated deficient cytotoxicity in our tested melanoma cell lines even at high concentrations of 100 to 400 µM. To be best of our knowledge, limited successes have been reported in the development of novel APE/Ref-1 inhibitors using structure-based approach. An important component of our study is to design, and screen small molecular inhibitors based on the APE/Ref-1 structure and their binding characteristics.

In our previous studies, the redox domain of APE/Ref-1 structure was selected for screening and molecular modeling. First, virtual screening of molecules from ICN database with over 3 million compounds was carried out using ICM software package developed by MolSoft L.L.C [36, 37]. The software incorporates ligand flexibility and ranks their binding using an energetic scoring function, which allows identification of top-ranked candidates. We further conducted cell-based bioactivity screening, and successfully developed our potent APE/Ref-1 inhibitor, R21; Bis-cinnamoyl-1,12-dodecamethylenediamine (N,N’-(dodecane-1,12-diyl)-bis(3-phenylacrylamide). Despite the superior anti-proliferation and anti-angiogenesis activity of R21 against several human malignancies, the compound had low water solubility, which we further improved the water solubility and successfully synthesized R21-OH; N-(12-cinnamamidododecyl)-3-(4-hydroxyphenyl) acrylamide. The viability assay conducted on our promising APE/Ref-1 inhibitor, R21-OH, evident its effective anti-melanoma activity at a concentration as low as 5 µM with improved water solubility.
To determine the binding activity of APE/Ref-1 with our promising inhibitor, R21-OH, and to further develop more bioavailable and potent inhibitors, we conducted crystallization study using purified truncated Δ40APE-1wt protein. Our successfully solved co-incubated structure failed to identify any binding of our potent compound, R21-OH, with Δ40APE-1wt protein; however, we did see the dimerization at cysteine 138 residue between two Δ40APE-1wt monomers. As detailed previously in our studies [13], the redox regulation of APE/Ref-1 is greatly affected under oxidative stress such as H₂O₂ or by cysteine replacement through site-directed mutagenesis. As such, our dimer structure obtained by co-incubation with our potent compound, R21-OH, can lead to a novel anti-tumor mechanism of APE/Ref-1. In support to our hypothesis, we then conducted crystallization of Δ40APE-1wt under oxidative stress (200 µM H₂O₂ + 10 µM Fe²⁺). Our solved structure form oxidized crystals also indicated the formation of a disulfide bond at cysteine 138 residue, which may greatly inhibit the redox regulation of APE/Ref-1.

We also conducted western blot experiment to determine whether our potent compound, R21-OH, facilitates dimerization of APE/Ref-1 using 10 mM TCEP (reducing reagent) as the negative control. As shown in this study, we found that monomer is the predominant form of Δ40APE-1wt under reducing condition, and when exposed to 10mM TCEP (reducing reagent), a conversion from dimer to monomer is evident. Our results from R21-OH incubation with the reduced Δ40APE-1wt also evident that our potent compound, R21-OH, facilitates dimerization of Δ40APE-1wt in a dose-dependent manner. Furthermore, we have identified the critical role of Cys138 in redox regulation of APE/Ref-1, as the predominant monomer form of Δ40APE-1C138A mutant protein was observed under oxidative stress and in the presence of R21-OH. The effect of cysteine 138 in APE/Ref-1 dimerization may help us explain the distinct redox regulation activity of APE/Ref-1, which may also provide new insight into the development of further novel inhibitors.
As explained in details earlier, studies on human melanoma cell lines have demonstrated the major role of APE/Ref-1 as an adaptive response to redox disequilibrium, which can promote cancer progression, and emerge drug resistance via maintaining its redox regulation and DNA repair activities [38, 79, 80]. It has been well established that the transcriptional activities of APE/Ref-1 are substantially affected by elevated levels of oxidative agents such as H$_2$O$_2$ and $'OH$ (ROS species) [81]. Induced levels of ROS, can generate oxidative DNA damage, which correlates with endonuclease and redox activity of APE/Ref-1 [81, 82]. Redox sensitive signaling of APE/Ref-1 can modulate gene expression of many transcription factors, including Nrf2 and STAT3 [80, 82, 83], and regulate intracellular redox state by inhibition of ROS production. Early pancreatic studies have demonstrated that the transcriptional activates of STAT3, is directly regulated by redox regulation of APE/Ref-1 [80], which can contribute to melanoma survival and proliferation [84, 85]. As such, many efforts have been implemented to target STAT3 signaling as a therapeutic strategy for melanoma treatment. Additionally, the antioxidant defense mechanism of Nrf-2 via mediate signaling on a set of antioxidant proteins via binding to antioxidant response elements (ARE) is well understood [39]. In our preliminary studies, we revealed the effect of Nrf-2 on antioxidant downstream target gene NQO1 expression via cells sensitize to H$_2$O$_2$ toxicity depletion, which significantly reduced APE/Ref-1 expression [12] to regulate and maintain redox homeostasis and cell survival [22]. Our pull-down analysis obtained from nuclear extraction of melanoma cells indicate a significant reduction of $\Delta$40APE-1$^{wt}$ binding to TFs such as Nrf-2 and STAT3 after H$_2$O$_2$ treatment. We also found that mutation of cysteine 138 ($\Delta$40APE-1$^{C138A}$) may completely knock down APE/Ref-1 binding to Nrf-2, and remarkably reduced binding to STAT3 in oxidative stress. Our results are suggesting that the redox status of APE/Ref-1 protein not only affects its dimerization but may also regulate its binding activity to TFs. To date, limited successes
have been reported in developing novel APE/Ref-1 inhibitors. The structural information collected by our study is critical to guide further design and synthesis of inhibitors targeting APE/Ref-1 to improve cancer treatment. The most potent inhibitor developed by our group using this structure-based approach showed promising anti-melanoma activities both in vitro and in vivo.
5 Conclusion

Apurinic/apyrimidinic endonuclease (APE)/Redox Factor-1 (Ref-1) was first recognized as a DNA endonuclease base excision repair (BER) enzyme [8] through cleavage of the phosphodiester bond at either the 5’ or 3’ end of an apurinic or apyrimidinic site [86]. APE/Ref-1 also serves as an essential redox regulation node of many nuclear transcription factors, such as Nrf-2, AP-1 dimers, Myb, NF-κB, p53, and HIF [8] in a redox-dependent manner, resulting in transcriptional activation of genes associated with cancer proliferation and progression. Many studies have proposed the major role of Cys65, Cys93, and Cys99 in the redox function of APE/Ref-1 [87, 88]. However, the structural studies of APE/Ref-1 show that these residues are not only buried in the core of the protein and not accessible, but they are also not in proximity to one another to participate in the formation of a disulfide bond [87, 88].
Figure 5.1. Human APE-1 structure (PDB: 5CFG, green) includes seven Cys residues. None of the cysteine residues is found forming disulfide bond within the molecule. A) Highlighted location of Cys65, Cys99, Cys310, and Cys208 residues at Δ40APE-1<sup>wt</sup>. B) Highlighted location of Cys65, Cys93, Cys99, Cys138, Cys208, and Cys296 of Δ40APE-1<sup>wt</sup>. C) Highlighted location of C138 of Δ40APE-1<sup>wt</sup>.

We also conducted a structural strategy to determine the binding profile of Δ40APE-1<sup>wt</sup> protein with our potent inhibitor, R21-OH, and to further develop APE/Ref-1 inhibitors for human melanoma therapy. Our structure study revealed that R21-OH facilitates the dimerization of APE/Ref-1 <i>in vitro</i>, which may be an innovative mechanism contributing to its anti-melanoma activity.
Additionally, we studied the effects of oxidation on APE/Ref-1 dimerization and redox regulation of nuclear transcription factors in human melanoma cell lines. We demonstrated that the redox environment not only affects the dimerization of APE/Ref-1 protein, but also regulates the interactions of APE/Ref-1 with nuclear transcription factors such as Nrf-2 and STAT3. For the first time, we demonstrated the structural transformation of the APE/Ref-1 protein under distinct redox conditions, which may contribute to its redox regulation of nuclear transcription factors. These novel observations pave the way for future drug development and optimization for melanoma therapy.

Figure 5.2 Possible mechanism of APE/Ref-1 redox regulation of nuclear transcription factors. As shown in the A) pathway, the reduced Δ40APE-1wt binds with nuclear transcription factors such as Nrf-2 by direct interaction through the active cysteine residue and subsequently activates the transcription of downstream target genes. However, under oxidative condition such as hydrogen peroxide (H₂O₂) exposure (as shown in B), the redox regulation of Δ40APE-1wt will form a dimer structure linked with Cys138-disulfide bond. Such dimer will no longer be able to bind and activate nuclear transcription factors, which may lead to inactivation of APE/Ref-1-mediated signaling.
References


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