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Perspective

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DNA Repair and Redox Activities and Inhibitors of Apurinic/Apyrimidinic Endonuclease 1/Redox Effector Factor 1 (APE1/Ref-1): A Comparative Analysis and Their Scope and Limitations toward Anticancer Drug Development

Miniperspective

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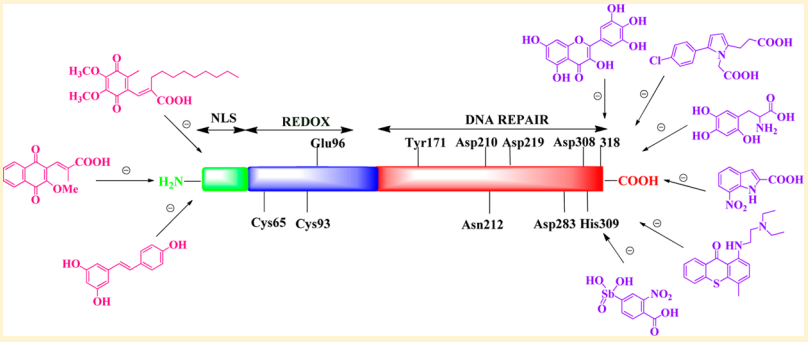

ABSTRACT: The apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1) is a multifunctional enzyme involved in DNA repair and activation of transcription factors through its redox function. The evolutionarily conserved C- and N-termini are involved in these functions independently. It is also reported that the activity of APE1/Ref-1 abruptly increases several-fold in various human cancers. The control over the outcomes of these two functions is emerging as a new strategy to combine enhanced DNA damage and chemotherapy in order to tackle the major hurdle of increased cancer cell growth and proliferation. Studies have targeted these two domains individually for the design and development of inhibitors for APE1/Ref-1. Here, we have made, for the first time, an attempt at a comparative analysis of APE1/Ref-1 inhibitors that target both DNA repair and redox activities simultaneously. We further discuss their scope and limitations with respect to the development of potential anticancer agents.

1. INTRODUCTION

The present state of cancer chemotherapy is that various factors lead to its failure through numerous mechanisms, which include the following: mutations in cancer cells, which alter the drug target; a reduction of cellular uptake of drugs due to various pharmacokinetic parameters; enhanced drug efflux by efflux transporters; drug detoxification by overexpressed drug metabolizing enzymes; blocked apoptosis; enhanced DNA repair; altered cell cycle checkpoints; resistance due to enhanced cellular stress responses; and the activation of microenvironment-induced drug resistance pathways.[1,2] Acquired resistance toward DNA-damaging agents may be due to the activation of the DNA repair system, which decreases the cell death caused by DNA-damaging chemotherapy.[3]

DNA-damaging agents can be endogenous, e.g., reactive oxygen species (ROS) produced during various metabolic processes, hydrolytic damage, nonenzymatic alkylation by *S*-adenosylmethionine, adduct formation resulting from the attack of reactive carbonyl species formed during lipid peroxidation, deamination and depurination (abasic, apurinic sites).[4] Various environmental insults, such as environmental hydrocarbon xenobiotics, are also responsible for DNA damage.[5,6] Exogenous agents that play a major role in damaging DNA are mainly ionizing radiation (IR), radiomimetic drugs, and DNA-damaging chemotherapeutic agents, such as anthracyclines, alkylating agents, platinum compounds, taxanes, and others.[7] All of these lead to abnormal base-pairing and the formation of defective proteins, which then leads to a cascade of mutations and finally cell death. Various endogenous DNA repair pathways naturally respond to DNA lesions and repair the damaged DNA.[8] These mechanisms include the following: repair of damaged bases and single strand breaks (SSBs) by the base excision repair (BER) pathway,[9]the repair of large adducts

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and bulky lesions by nucleotide excision repair (NER), recognition and repair of mismatches or misalignments of short nucleotide segments by mismatch repair (MMR), direct repair of alkyl adducts by O6-methylguanine-DNA methyltransferase (MGMT), the repair of double strand breaks (DSBs) by homologous recombination (HR); and nonhomologous end joining (NHEJ).[3,10,11] Each of these pathways employs various specialized sets of enzymes and proteins.[12−16]

It has been reported that 2000−10 000 AP sites are created every day in each human cell by hydrolytic depurination, and these sites are subsequently repaired by the BER pathway.[4,17−19] Human cells have two types of genomes, nuclear and mitochondrial, which are continuously under the effect of both exogenous and endogenous factors. Because of the dynamic nature of both genomes, they have the ability to respond to changes and adapt.[20] It has been found that environmental factors and normal metabolic processes lead to ∼1000−100 000 molecular lesions per cell per day in humans.[21] The sources of these factors are endogenous (misincorporation of uracil, deamination of cytosine, hydrolysis of the four bases, oxidation by ROS and reactive nitrogen species (RNS), and alkylation of bases by lipid end products) and exogenous (radiation, such as UV, X-ray, and IR, and xenobiotics). The most common damage from these factors is from SSBs.[20−23] SSBs arise mostly because of oxidative attack of endogenous ROS on DNA.[24] Mitochondria are a major source of free radicals, resulting in DNA instability; hence, they are more prone to mutations compared to nuclear DNA.[20] Many of these DNA defects are rectified by the BER pathway, which was discovered by Thomas Lindahl in 1974 and is conserved from bacteria to mammals.[25,26]

Certain cancers are deficient in one DNA repair pathway (e.g., HR) but have an alternative pathway that remains functional, e.g., the BER pathway. It has also been found that repair proteins are overexpressed in various cancers. Therefore, DNA repair pathways can be targeted selectively, and innovative anticancer therapy can be developed from various repair protein inhibitors.[3] The BER pathway is a major target because several proteins of this pathway lead to embryonic lethality in mouse models.[27,28] Apurinic/apyrimidinic endonuclease (APE1) and polymerase β (Polβ) are found to be the key players in the BER pathway. No effective backup to the functions of APE1/Ref-1 has been observed in mammalian cells.[10] It alone is responsible for 95% of the endonuclease activity on DNA in the BER pathway because it is a multifunctional enzyme.[29] The C- and the N-termini of APE1/Ref-1 are involved in two different and independent functions, which collectively lead to enhanced cell survival by repairing damaged DNA. They also promote transcriptional activation that encourages cell growth by expression of various growth-promoting genes.

2. APE1/Ref-1

There are two classes of AP endonucleases, class I and class II (Figure 1). Class II can be further classified into two families, which differ in structure but have the same functional mechanism. These families are classified from their structural similarity to two endonucleases in *E. coli*, i.e., exoIII and endoIV. APE1/Ref-1 is the human homologue of exoIII, with 26% sequence identity. APE2 is also an exoIII homologue and has yet to be fully characterized.[30]

APE1, a 318-amino acid, 35 000 Da globular protein, consists of two distinct domains: the N-terminus and C-terminus. Each

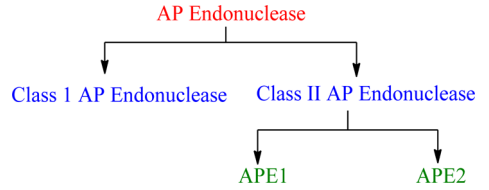

Figure 1. Classification of AP endonucleases.

domain is composed of six-stranded β sheets surrounded by α helices, which collectively form a four-layered α/β sandwich fold. The interior comprises antiparallel β strands, while the α helices line the exterior.[31,32] The first crystal structure of APE1 was reported in 1997 (PDB code 1BIX), as shown in Figure 2.[32]

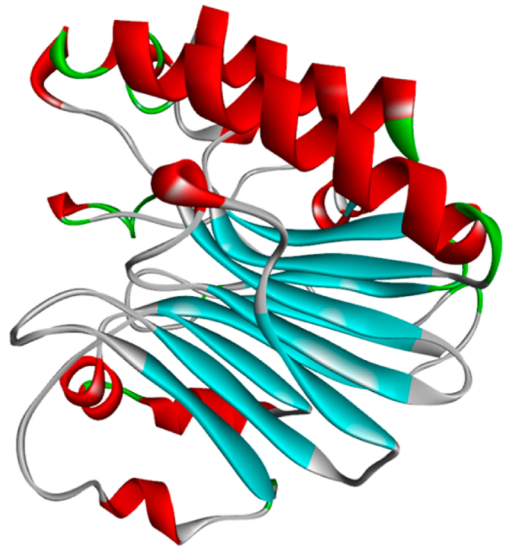

Figure 2. Crystal structure of APE1 (PDB code 1BIX) visualized using Accelrys Discovery Studio Visualizer 3.5.

APE1 was first recognized as human APE1 (HAPE) by Demple and colleagues in 1991.[29] The first 33−35 amino acids (Figure 3) of the N-terminal domain are involved in protein−protein interaction(s) and RNA binding activity.[33] It plays an essential role in the transfer of cytoplasmic APE1 to the nucleus under oxidative stress conditions.[34] The amino acids 161−318 in the C-terminal regions are responsible for DNA repair activity by the BER pathway.[35] In 1992, Xanthoudakis and Curran reported that APE1 stimulates the DNA binding activity of the transcription factor (TF) AP-1 through conserved cysteine (Cys) residues in c-Fos and c-Jun.[36] It was further demonstrated that the N-terminal domain (the region between amino acids 35 and 127) plays a major role in the redox regulation of various TFs (e.g., AP-1, NF-κB, CREB, p53, HIF-1α, STAT3, and Pax-6). Such regulation contributes to cell growth and progression, angiogenesis, inflammation, down-regulation of apoptosis, etc. in malignant melanomas.[37,38] APE1 converts inactive TFs to their reduced active form, enhances their DNA binding, and increases the expression of various genes responsible for progression and promotion of tumors.[10,39] Therefore, it was named redox effector factor 1 (Ref-1). The two main functions of this protein are independent.[35,40,41]

2.1. Regulation. The APE1 protein is encoded by the *APE1* gene located on chromosome 14. The gene is 2.6 kb in size and

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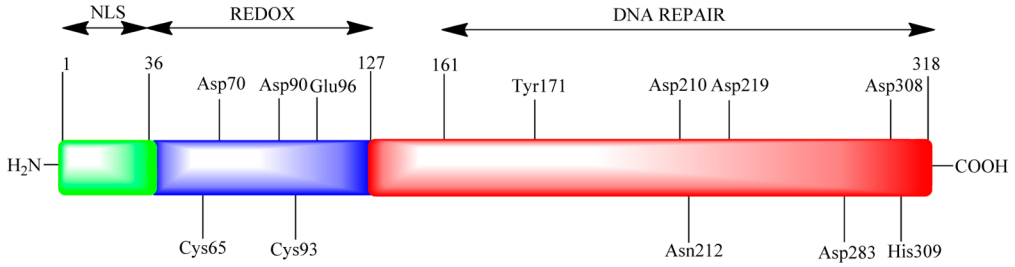

Figure 3. Representation of amino acid residues involved in endonuclease and redox activity of APE1.

contains four introns and five exons, with the first being noncoding.[42] Gene expression is regulated at the transcriptional and post-transcriptional level. It has been observed that hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) induce expression of the *APE1* gene.[41,43] Oxidative stress can also induce *APE1* expression.[44−48] The ultimate outcome and activation are illustrated in Figure 4. Oxidative stress also

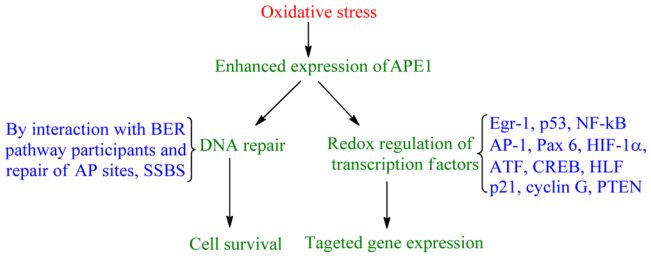

Figure 4. Multifunctional role of APE1 in response to oxidative stress. Transcription factors are early growth response protein 1 (EGR-1), nuclear factor κ light-chain enhancer of activated B cells (NF-kB), activator protein 1 (AP-1), paired box protein (Pax 6), activating transcription factor (ATF), cAMP response element-binding protein (CREB), hepatic leukemia factor (HLF), phosphatase and tensin homologue (PTEN).

enhances nuclear translocation of thioredoxin (TRX), which is an important factor for activation of the Cys residues of APE1, which are responsible for the redox function. External stimuli, such as cytokines and hormones, are also responsible for regulation of APE1 expression.[41] It has an inverse relationship with p53, which downregulates APE1 expression to follow another path toward p53-dependent DNA damage-induced apoptosis.[47,49] APE1 activates p53 and stimulates its DNA binding.[50] APE1 activates only wild-type p53 for enhanced DNA binding, but it does not affect mutated p53.[51,52] APE1 plays an essential role in p53-mediated apoptosis. It has been found that the antiapoptotic protein Bcl2 directly interacts with APE1 and inhibits the BER pathway by downregulating the *APE1* gene.[41,53]

Helicobacter pylori (*H. pylori*) infection is also an inducer of APE1.[54] The activation of APE1 inhibits gastric epithelial cell (GEC) apoptosis by inducing cell proliferation and causing DNA repair of damaged cells.[55] Various stimuli and their effects have been described in Figure 5.

2.2. Location of APE1. APE1 is abundantly expressed in many cell types, as many as 10^5−10^6 molecules per cell, and has a relatively long half-life of 8 h.[56] Nuclear expression of APE1 is observed in stromal and epithelial ovarian, adrenal medullary, cervical basal, and parathyroid glandular epithelial cells, possibly because of its DNA repair function.[57,58] APE1 is also highly expressed in many selected regions of the central nervous

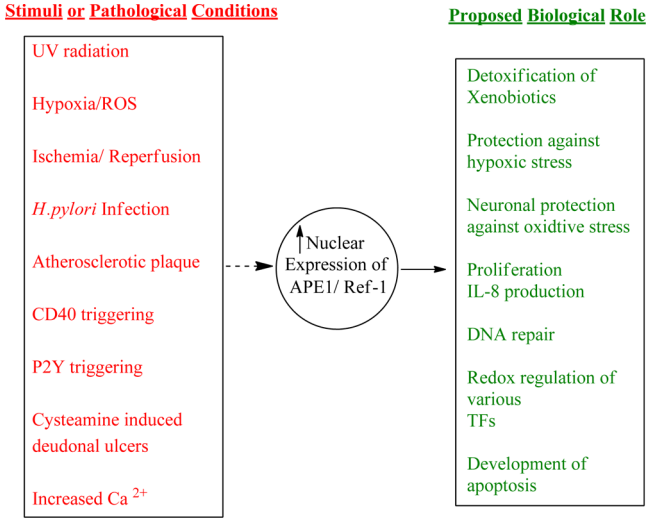

Figure 5. Stimuli and biological roles of APE1.

system (CNS).[59,60] It is found predominantly in the dentate gyrus, CA3 and CA4 regions of the hippocampus.[61]

APE1 is highly expressed in the cytoplasm of many cells, including macrophages, spermatocytes, hippocampal cells, hepatocytes, hypoglossal motor neurons, and breast cells.[58,61,62] APE1 protein is found in the cytoplasm, where it maintains newly synthesized TFs in a reduced state, which is their active form. It has been observed that APE1 localizes to the nucleus in response to oxidative stress.[34] In some cells, such as adrenocortical, cerebellar Purkinje, pneumocytes, some cervical cells, and parietal and mucosal cells of the stomach, both cytoplasmic and nuclear localization of APE1 has been observed.[42,63] Higher levels of APE1 are found in cytoplasm than in the nucleus.[64] The expression of APE1 has also been found in human and ascidian gametes and embryos.[65]

APE1 is scarce in mitochondria; therefore, targeting mitochondrial DNA (mtDNA) repair is an important therapeutic target for maintaining neuronal cell genome integrity.[19] Mitochondrial DNA is 10−15 times more susceptible to oxidative damage because of the absence of histones and other proteins, which are capable of reducing oxidative damage. mtDNA is located in proximity to the electron transport chain (ETC) and is more exposed to high levels of ROS. Additionally, the NER is absent in mitochondria, so the BER pathway is the major pathway for mtDNA repair involving APE1.[18,19,56]

3. DNA REPAIR ACTIVITY

DNA repair activity is localized to the C-terminal region of APE1. As discussed above, the major pathway involved in repair of oxidized base damage, AP sites, and strand breaks is the BER

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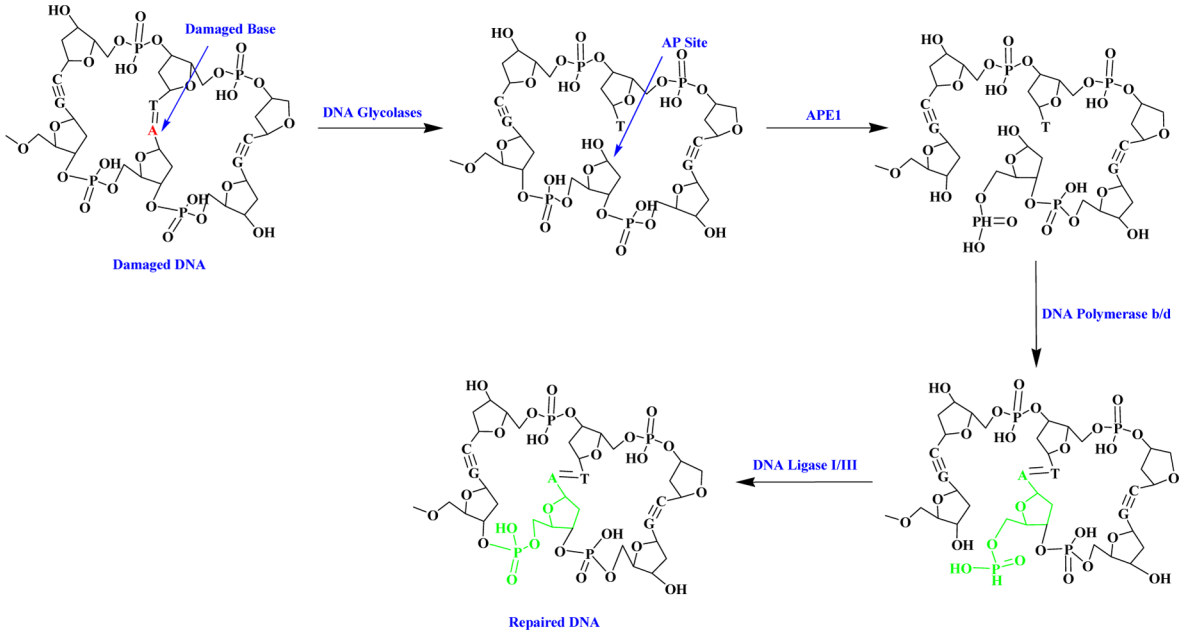

Figure 6. Steps and key enzymes involved in the BER pathway.

pathway. The BER pathway involves two subpathways for repair, i.e., short patch (SP) and long patch (LP), which differ in the involvement of different proteins as well as the size of the damaged DNA involved in the repair. The mechanism, as shown in Figure 6, first involves removal of damaged bases by hydrolyzing the *N*-glycosidic bonds with the DNA glycosylase enzyme. In SP-BER, only one defected base is removed, whereas in LP-BER, three to eight damaged bases surrounding an AP site are removed by glycolases. This leaves the DNA backbone intact with an AP site but with the absence of bases. APE1 acts on an AP site and hydrolyzes the phosphodiester bond, which is immediately 5′ to the AP site. This leaves a free 3′-OH group and a deoxyribose 5-phosphate on an AP site. Subsequently, other enzymes act to repair the cleaved site. Polymerase β replaces the damaged nucleotide with a new nucleotide, and DNA ligase seals the nicks.[17−19,66,67]

3.1. Mechanism of Endonuclease Activity. *3.1.1. Key Amino Acids Involved in the Endonuclease Activity of APE1.* The active site for endonuclease activity in APE1 is in the form of a V-shaped pocket, and it lies at the top of the α/β sandwich (Figure 7). It has two sites for metal ion binding, A and B.[30,68,69] The active site compriseds several amino acid residues (His309, Glu96, Asp283, Thr265, Tyr171, Asn68, Asp210, Asp70, Asp90, and Asn212), which are essential for endonuclease activity.[68] Phe266 is not essential for the incision activity of APE1 but is involved in the recognition of the AP site.[70] APE1 can incise acyclic AP sites in the absence of Mg^{2+}.[71] Various ions, including arsenic [As(III)], cadmium [Cd(II)], cobalt [Co(II)], iron [Fe(II)], nickel [Ni(II)], lead [Pb(II)], and Fe, as well as CO, have been shown to inhibit APE1 activity,[72] confirming the relationship between many environmental metals and cancer. Arg177 participates in binding through the major groove, and Met270 through the minor groove; both effectively lock APE1 to abasic DNA.[68,73]

3.2. Moving Metal Ion Mechanism. At least one divalent ion (Mg^{2+}) is required for the endonuclease activity of APE1.[67] It has also been proposed that two divalent metal ions are present in the crystal structure of APE1.[74] Some high-resolution crystal structures of APE1 in complex with DNA or alone have

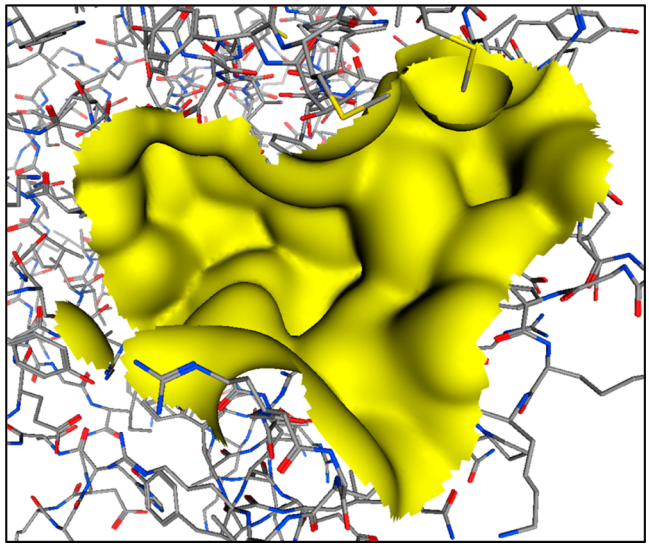

Figure 7. Ribbon structure of APE1 showing the V-shaped endonuclease active site (PDB code 1BIX).

shown different positions of one or two metal ions in the active site, depending on the pH. Crystal structures with low pH, such as 1DE9 and 1HD7, have one metal ion in the active site (A site), whereas an additional Pb^{2+} ion is present in the neutral structure 1E9N in the more buried site (B site). In a "two metal" cleavage reaction, one metal ion stabilizes the attacking nucleophile in coordination with Asn212, Asp210, and His309 in the B site (Figure 8, scheme 3), while the other metal ion stabilizes the leaving group in the transition state by coordinating with Glu96, Asp70, or Asp308 in the A site. Both sites cannot be occupied simultaneously by two metal ions because the enzymatic activity decreases at high metal ion concentrations. Oezguen et al. proposed that there is only one metal ion present in the active site of APE1, which moves between site A and site B during and after the catalytic reaction to facilitate substrate cleavage. The movement of the metal ion depends on the net charges of the active residues in the cavity.

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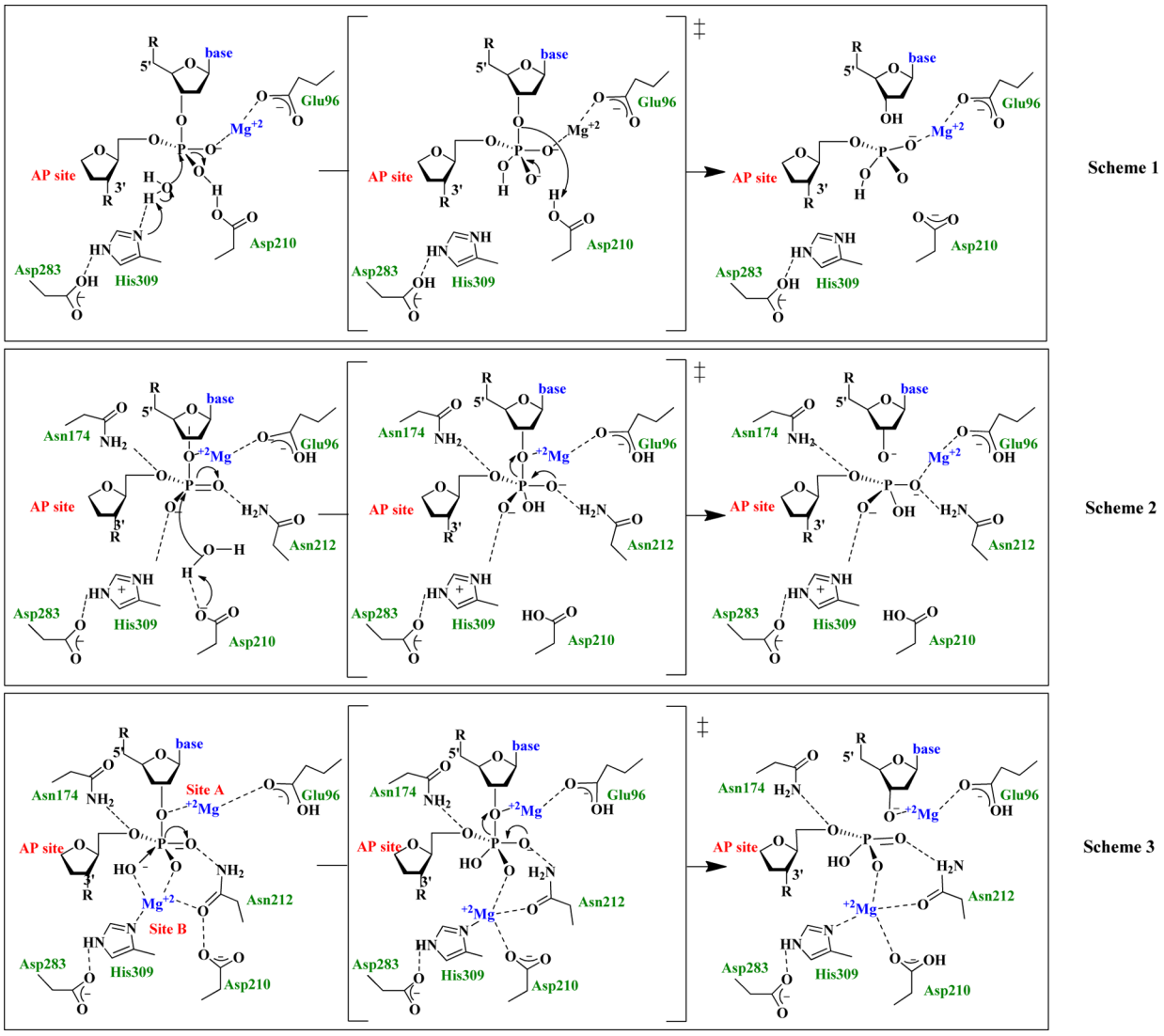

Figure 8. Catalytic mechanism(s) of endonuclease activity of the active site of APE1.

When His309 and Asp210 are individually protonated, metal ion movement between the two sites does not take place.[69,74−76] Glu96 is also found to coordinate the metal ion in both A and B sites and move with the metal during simulations.[69]

Mutation of Glu96 to Ala has been reported to cause a decrease in activity up to 600-fold, showing that Glu96 plays a critical role in the catalytic function of APE1.[77] Glu96 is the residue that directly coordinates with Mg^{2+}. Three water molecules are required in the active site to accomplish the catalytic activity. Asn68, Asp70, and Asp308 coordinate with the water in the magnesium−water cluster. Mutation of Asn68 reduces the endonucleolytic activity by 200-fold, whereas mutation of Asp70 and Asp308 reduces activity by 25- and 5-fold, respectively.[78] Similarly, mutations in Thr171 and Asp210 showed 5000-fold and 25 000-fold less activity, respectively.[71] It is proposed that Asp210 serves as a proton donor and Tyr171 interacts with Glu96 to form the active site interacting with DNA. The Asn212 mutant also failed to bind to the AP site, showing its necessity in the catalytic activity.[42,68] Mutation in the Phe266 residue of APE1 resulted in a 6-fold decrease in both specificity and binding affinity, and mutation of Trp267 also resulted in a 6-fold reduction of DNA binding affinity.[70]

His309 and Asp283 are believed to play a key role in the catalytic activity of APE1 (Figure 8, scheme 1). Uncharged His309 acts as a base and abstracts a proton from a water molecule. Asp283 stabilizes the His309 key residue for endonuclease activity. The resultant hydroxide ion acts as a nucleophile and cleaves the DNA strand at the 5′ end. The metal ion polarizes the transition state and stabilizes the negatively charged oxygen atom, as shown in Figure 8 (scheme 1).[71,75] In another proposed mechanism, Asp210 activates the attacking water by abstracting the proton and forming a nucleophilic hydroxide ion, whereas the positively charged His309 facilitated by Asp283 polarizes the negatively charged oxygen (Figure 8, scheme 2). The Mg^{2+} ion coordinates with the phosphate group and stabilizes the transition state. Asn212 also plays a major role in the stabilization of the intermediates and cleavage of the deoxyribose phosphodiester.[68]

4. REDOX ACTIVITY

APE1/Ref-1 converts the inactive oxidized forms of various stress or hypoxia-induced TFs, such as AP-1, NF-κB, ATF/CREB, p53, Pax5, Pax8, c-Myb, HIF-1α, and Egr-1,[10,36,39,41,48,50,79−84] to their reduced active form, thereby enhancing their DNA binding activity. Various genes are

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expressed that mediate genomic stability and are involved in cancer enhancement and progression by promoting proliferation and angiogenesis and downregulation of apoptosis (Figure 9).

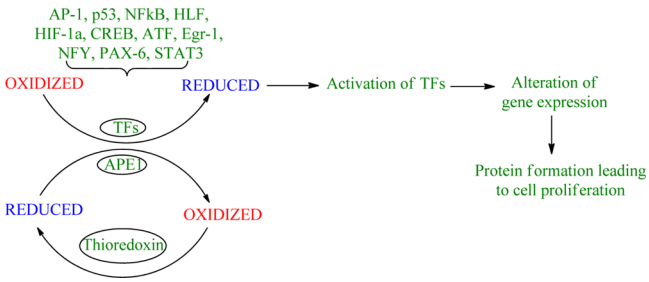

Figure 9. Mechanism of redox regulation of various TFs by APE1/Ref-1.

4.1. Keys Amino Acids Involved in the Redox Function of APE1/Ref-1. The N-terminal amino acids 35−127 of APE1/Ref-1 are involved in its redox function. This region lies as an extended loop across β strands. Seven conserved Cys residues are found in human APE1: Cys65, Cys93, Cys99, Cys138, Cys208, Cys296, and Cys310. The redox function of APE1 is observed only in mammalian APE1, whereas the zebrafish (zAPE1) homologue lacks this function. In addition, zAPE1 contains only five Cys residues compared to the seven Cys residues present in mammalian APE1.[85] Cys65 and Cys138 are not conserved in zAPE1.[85,86] Cys65 is necessary for the full redox function of APE1.[85,87−89] Any mutation in the Cys65 residue decreases redox activity.[48]

4.2. Redox Mechanism. APE1 reduces the Cys residues of TFs, making them active for further gene activation processes.[41] The Cys65 residue is found on the first β strand, which is in the core of the protein, with its side chain pointing toward the hydrophobic pocket and away from the central β strand sheet. Cys65 is buried in the protein and is not directly solvent-accessible. Therefore, direct involvement of Cys65 in the reduction process is an as yet unresolved issue.

It is hypothesized that Cys65 forms a disulfide bond with the Cys93 residue.[90] However, they are distant from each other (9 Å), whereas a distance of 2.2 Å is required for disulfide bond formation. Both of these residues are also present on opposite sides of the β sheets. Other residues, such as Cys99 and Cys138, are solvent-accessible but not close enough to form a disulfide bond. Furthermore, their mutation to Ala does not cause the loss of redox activity, which implies that they do not play a key role in the redox function.[10] However, a mutation of Cys99 to Ser has shown altered activity of the enzyme binding to an AP site.[91] A conformational change may be taking place to allow Cys65 to move in the vicinity of Cys93 and form a disulfide bond, which is the oxidized form. Thioredoxin (TRX), a redox protein, reduces this disulfide bond and makes the Cys65 redox active, as illustrated in Figure 10.[92,93] Cys65 further reduces the disulfide bond of TFs and again retains the form of a disulfide.[10]

The Cys32 of TRX mediates a nucleophilic attack on an oxidized disulfide linkage. The mixed disulfide formed is resolved by Cys35, resulting in the formation of a disulfide bond in TRX. This reduces Cys65 and Cys93 in APE1/Ref-1 and oxidizes itself, as shown in Figure 10. The reduced form of APE1 is the active form. This activated, reduced Cys65 then reduces the disulfide bond of various TFs and converts them to their reduced active form to further perform their functions, e.g., activation of target gene expression, cell growth, and proliferation.

5. APE1 AND CANCER

The relationship between cancer cells and APE1 levels is of great importance. First, APE1 plays an important role in the proliferation of various cancer types. Second, APE1 is also useful in developing diagnostic markers for early cancer detection.

The elevated and altered expression of APE1 has been found in various cancers, such as prostate,[94] osteosarcoma,[95] breast,[96] bladder,[97] cervical,[98] colorectal,[99] lung,[100] ovarian,[101] pancreatic,[102] gastroesophageal, and pancreaticobiliary cancers.[103] APE1/Ref-1 is highly expressed in non-small-cell lung cancer (NSCLC), and higher levels of serum autoantibodies of APE1 in NSCLC act as tumor markers.[104] Oxidative stress contributes to the induction of APE1 in the lung carcinoma cell line H460.[105] APE1 expression is elevated in prostate cancer,[94] and elevated activity is observed in human glioma.[106] Furthermore, APE1 is overexpressed in both the nucleus and cytoplasm of human hepatocellular carcinomas (HCC).[107] Cytoplasmic expression of APE1 has been found to increase in HCC patients.[108] Cytoplasmic APE1 also causes expression of cycloxygenase 2 (COX2) via activation of NF-κB and promotes lung tumor aggressiveness.[100] APE1 enhances cell survival, with reduced ROS-generated oxidative stress being observed in irradiated A549 lung cancer cells, and protects these cells from apoptosis.[109] In malignant and premalignant cervical,[110] prostate, ovarian, epithelial,[111] and colon cancers, as well as rhabdomyosarcomas, osteosarcomas,[95] and germ cell tumors, APE1 expression is dramatically elevated compared to normal tissues.[37] In other types of cancers, APE1 levels are unchanged, but their distribution between normal and cancer cells is altered. In ovarian and colon cancers, there is also altered expression of APE1, but levels are elevated at the same time.[42,111] The proto-oncogene *c-Myc*, involved in the development of various cancers, has been found to be cleaved by APE1.[112] The tumor microenvironment, characterized by low oxygen, low nutrients, and low pH, plays an important role in the elevation of APE1 activity, as observed in colon carcinoma RKO cells. APE1-downregulated RKO cells grown at pH 7.4 have no change in survival compared to control RKO cells, but survival is lower compared with controls at pH 6.0.[113] Telomere ends are crucial for the aging of cells. The N-

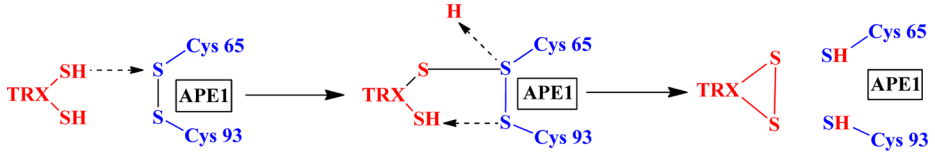

Figure 10. Reduction and activation of oxidized Cys residues in APE1/Ref-1 by TRX.

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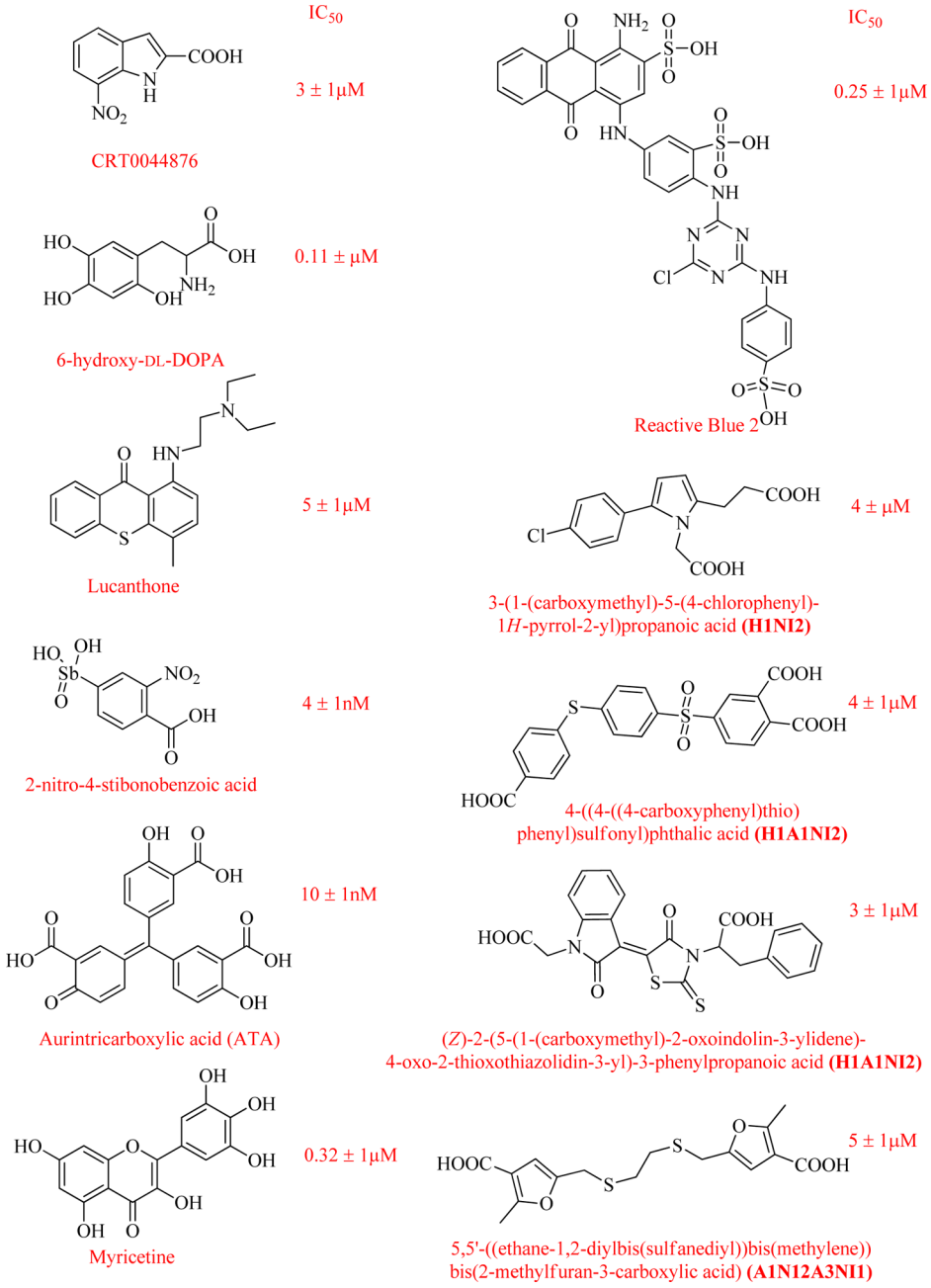

Figure 11. Various scaffolds of endonuclease inhibitors of APE1.

terminal domain of APE1 has been found to stabilize the telomere ends, and its depletion causes dissociation of the telomeric repeat-binding factor 2 (TRF2) protein from telomeres, as observed in U2OS osteosarcoma cells, BJ-hERT fibroblast cells, and HeLa cervical carcinoma cells.[114] The guardian of the genome, the tumor suppressor protein p53, physically interacts with APE1 and is involved in the alteration of APE1 activity in cadmium-induced stress in HCT116 human colorectal cancer cells. APE1 polymorphisms have been associated various cancers. A total of 18 polymorphisms have been reported for APE1. Among these, the Asp148Glu polymorphism is the most extensively studied. Meta-analysis studies showed that APE1 Asp148Glu is not associated with breast cancer susceptibility in the Asian population[115] and not associated with lung cancer or colorectal susceptibility among the Asian or Caucasian populations.[116] A statistically significant association has been found for the APE1 Asp148Glu mutation and an increased risk of lung cancer (3.17-fold) in smokers.[117] In the Chinese population, the APE1 genetic variant rs1760944 has been associated with gastric cancer risk development.[118] Post-translational modifications play an important role in cancer development, and it has been found that deregulated APE1 acetylation status has been observed in triple-negative breast cancers.[119] The breast cancer susceptibility gene *BRCA1* stimulates the expression and activity of APE1/Ref-1 in association with octamer-binding transcription factor OCT1.[120] APE1/Ref-1, via its redox activity, regulates the interaction of estrogen receptor α with estrogen response elements, the estrogen-responsive progesterone receptor and pS2 genes. Lou et al. reported on (2*E*)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid (E3330) as a redox inhibitor of APE1 in 2008. E3330 limits ERα-ERE

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complex formation in vitro in MCF-7 breast cancer cells.[121] E3330 blocks fatty acid induced and tumor necrosis factor α (TNF-α) induced activation of IL-8 production, which is involved in inflammation, a characteristic of cancer progression, in liver cancer cell lines. E3330 is also involved in the inhibition of pancreatic cancer development via inhibiting DNA binding to the APE1-regulated TF STAT3.[122]

The overexpression of APE1, and its repair and redox activities, has been associated with tumor cell resistance against alkylating agents and radiotherapy.[123,124] Impaired APE1 enhances the cellular sensitivity of alkylating agents and antimetabolites during tumor treatment.[125] APE1 potentiates the activation of the multidrug resistance (MDR1) gene by regulating YB-1. Inhibition of APE1 causes growth reduction in SKOV-3x ovarian cancer cells[101] and enhances radiosensitivity of hepatocellular carcinoma cells.[126] Several DNA repair mechanisms involved in safeguarding the genome can facilitate drug resistance and cancer cell survival by removing chemotherapy-induced DNA adducts in glioblastoma multiforme (GBM).[127] Oxidative stress elevates APE1 levels, which further contributes to the resistance to alkylating agents used in adjuvant chemotherapy for brain cancer, such as GBM.[128] The cancer stemlike cell subpopulation in GBM also contributes to treatment resistance.[127] All-trans-retinoic acid (ATRA) induced chemoresistance in myeloma cells has been observed to be due to upregulation of APE1 via a noncanonical signaling pathway. This leads to an enhanced prosurvival activity counteracting melphalan (an alkylating agent).[129]

APE1 is a potential target for enhancing radiosensitivity in glioma cells. An in vitro and in vivo study in HCC indicates that downregulated APE1 enhances radiosensitivity and potentiates the growth, proliferation, and apoptosis induction of human HCC cells.[124]

Several studies have shown that depletion of APE1 leads to sensitization of tumor cells to DNA damage, abolishes the activity of gene products regulated by various TFs, and ultimately leads to the death of cancer cells.[101,130,131] Antisense depletion of APE1 is found to hypersensitize HeLa cells to alkylating agents, such as methyl methanesulfonate (MMS), H_2O_2, menadione, and paraquat. Antisense APE1 downregulation also hypersensitizes lung cancer cells to radiotherapy, pancreatic cancer cells to gemcitabine,[132] and glioma cells to MMS, temozolomide (TMZ), and nitrosoureas. siRNA-mediated APE1 depletion in osteosarcomas also enhances cytotoxicity to alkylating agents and H_2O_2.[30]

Various inhibitors have been identified that inhibit the cleavage of AP sites in vivo in SF767 glioblastoma cells, and ARO3 has been found to have potential to inhibit the growth of cancer cells.[133] Various APE1 inhibitors have been developed for the treatment of cancer, such as lucanthone, the derivative hycanthone,[134] 6-hydroxy-DL-DOPA, Reactive Blue 2, myricetin,[135] (2*E*)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid, E3330,[136−138] the E3330 analogs RN8-51, RN10-52, and RN7-60,[139] and methoxyamine (MX).[140] These inhibitors downregulate the altered APE1 expression and activity in tumor cells and enhance tumor sensitivity to radiotherapy and chemotherapy. The development of small molecule inhibitors of APE1 may lead to the attenuation of APE1 activity at the N- or C-terminal, and APE1 may prove to be a potential therapeutic target in the treatment of resistant cancers.[141]

6. APE1 INHIBITORS

As endonuclease activity of the C-terminus and redox activity of the N-terminus of APE1 are independent, different inhibitors are reported for their active sites.

6.1. Inhibitors of Endonuclease Activity. Any compound that can halt the BER pathway causes an accumulation of AP sites. Because AP sites are more susceptible to DNA-damaging anticancer agents, this further damages cancer cells and leads to cell death. Thus, the damage of DNA in cancer cells due to various drugs is much more ensured.[73] Many compounds have been successfully identified as repair inhibitors from random screenings of compound libraries. In silico screening of compounds has led to the identification of several DNA repair inhibitors from pharmacophore models developed on the basis of the interaction of AP DNA with active site residues.[142] The most potent inhibitors are known to have a pharmacophoric scaffold similar to the 3′- and 5′-deoxyribose-phosphate scaffold of AP DNA.[11,30,134,143] APE1 inhibitors synthesized and studied so far are illustrated in Figure 11, along with their IC_{50} values. These inhibitors act via two mechanisms, which are tabulated in Table 1.

Table 1. Specific and Nonspecific DNA Repair Inhibitors

sr. no.	specific DNA repair inhibitors	nonspecific repair inhibitors
1	CRT0044876	methoxyamine
2	6-hydroxy-DL-DOPA	
3	myricetine	
4	lucanthone	
5	arylstibonic acids	
6	aurintricarboxylic acid (ATA)	

(1) Specific (direct) inhibitors interact with APE1 and inhibit it from binding to the abasic DNA (i.e., AP) site.[144]
(2) Nonspecific (indirect) inhibitors bind to the AP site on DNA and form a stable adduct. They inhibit APE1 binding to the AP site and display endonuclease activity.[143,145]

From the screening of 5000 compounds, Madhusudan et al. reported the first biological and biochemical endonuclease inhibitor of APE1. Compound **1** (CRT0044876) (7-nitroindole-2-carboxylic acid) was found to be a potent and selective inhibitor of APE1 and enhanced the cytotoxicity of MMS in vitro. However, this compound was not developed further as a lead compound because of its poor druglike properties and toxicity issues due to the nitro aromatic group. In another study, Simenov et al. proposed the novel inhibitors 6-hydroxy-DL-DOPA (dihydroxyphenylalanine), aurintricarboxylic acid (ATA), myricetin, and Reactive Blue 2 (Figure 11) from screening of a library of pharmacologically active compounds (LOPAC). ATA was found to be the most potent, and it enhanced the cell-killing effect of alkylating agents, such as methoxyamine, 6-hydroxy-DL-DOPA, myricetin, Reactive Blue 2, and methyl methanesulfonate. However, ATA showed additional off-target effects and suffered from being unstable in the presence of oxygen.[146]

Seiple et al., from the high-throughput screening of 2000 compounds, suggested that arylstibonic acids can be employed as useful scaffolds for the design of endonuclease inhibitors (Figure 11). Compound 13755 was reported to be the most potent repair inhibitor of APE1.[147] These compounds were

found to have whole-organ toxicity potential due to the heavy metal antimony.[73]

Zawahir et al., in 2009, designed a set of pharmacophoric models based on the interaction of abasic DNA with APE1. A total of 365 000 molecules were screened on the basis of these models. The best hits (Figure 11) were found from the models H1NI2, H1A1NI2, and A1NI2A3NI (H denoting hydrophobic moiety, NI denoting negatively ionizable group, A denoting a hydrogen bond acceptor). These were the first rationally designed inhibitors of the AP endonuclease.[142]

Methoxyamine was used in an assay to detect AP sites in vitro in the BER pathway.[148] Later, Taverna et al. and Fischel et al., in their independent studies, reported that methoxyamine is an indirect inhibitor of APE1 and that it further enhances the cytotoxic effect of TMZ in colon and ovarian cancers.[131,148,149]

6.2. Redox Inhibitors. Inhibiting APE1's redox activity appears to be a promising target in the inhibition of cancer cell growth and promotion of cancer cell death. Various TFs, such as HIF-1α, p53, NF-κB, CREB, and AP-1, have been explored in the important stages of cancer, including angiogenesis, progression, and promotion of tumor cell growth.[150−154] By inhibiting APE1's redox activity, these TFs cannot bind to their consensus DNA to induce gene expression and tumor-related uncontrolled growth and signaling pathways. Various small molecule redox inhibitors, capable of attenuating one or more functions, offer potential for the reversal of drug resistance.[11,155] The list of various redox inhibitors of APE1 is given in Figure 12.

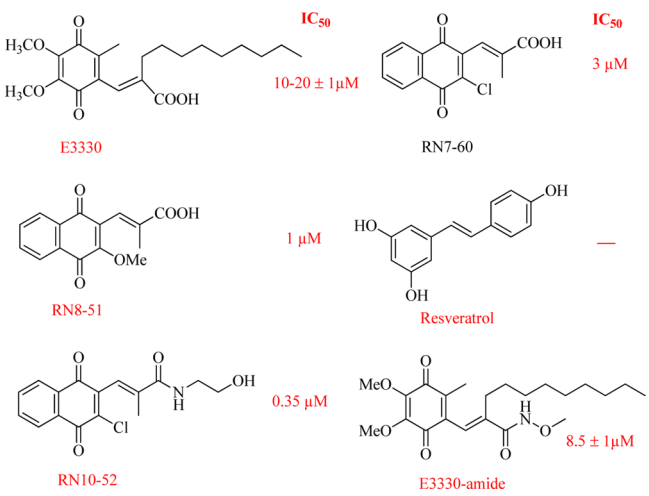

Figure 12. Various scaffolds of redox inhibitors of APE1.

E3330 checks the growth of cancer cells by inhibiting cell proliferation and angiogenesis.[85] Manvilla et al. proposed a mechanism for redox inhibition in which E3330 binds specifically to the repair active site of APE1 used for stabilization of the enzyme for redox activity and prevents it from undergoing a conformational change. According to this study, E3330 shows redox inhibition in the unfolded state of APE1. The inhibition of endonuclease activity of APE1 was also observed at high concentrations of E330.[137]Amidation of E3330 was performed by Zhang et al. to confirm whether the carboxylate of E3330 is essential for redox function. They found that E3330-amide (IC_{50} = 8.5 μM) was a more effective redox inhibitor than E3330 (IC_{50} = 20 μM) of AP1-DNA binding assisted by APE1. In addition, E3330-amide did not show any binding to the repair active site, which proves that the carboxylate is essential for binding to the repair active site and that the quinone moiety is essential for redox inhibition.[156] Nyland et al. synthesized various benzoquinone and naphthoquinone derivatives of E3330. Various quinone analogs were analyzed by Kelley et al., as shown in Figure 12. RN8-51, RN10-52, and RN7-60 were found to be the best hits with the lowest IC_{50} values.[157]

Another study by Raffoul et al. (2007) reported that soy isoflavones downregulate redox activity as well as the APE1 enzyme in prostate cancer cells, both in vitro and in vivo.[158,159] An earlier study by Yang et al. (2005) reported that resveratrol is involved in inhibition of the redox function of APE1, resulting in reduced DNA binding of AP1 and NF-κB TFs, which play major roles in cancer progression.[160] At high concentrations, it was also found to inhibit APE1 endonuclease activity. However, these results have not been supported by others or shown to be effective at physiological levels.[10]

7. CLINICAL STATUS

Only two APE1 inhibitors are currently in clinical trials approved by the U.S. Food and Drug Administration (FDA).

(1) Lucanthone: Due to its high lipophilicity, it can easily cross the blood−brain barrier (BBB). It is in phase II clinical trials for the treatment of brain metastases from non-small-cell lung cancer as an adjuvant to radiotherapy. It is required at much lower doses clinically compared to that used in vitro studies.[73,134]

(2) Methoxyamine: It is currently in phase I clinical trials being conducted by TRACON Pharmaceuticals. It is being studied in combination with TMZ for the treatment of advanced or metastatic solid tumors, primary brain tumors, lung cancer, and various other malignancies. A significant preclinical data set from the Gerson laboratory reveals that methoxyamine increases the cytotoxicity of various alkylating agents, such as TMZ and carmustine, significantly.[73]

8. WHICH DOMAIN OF APE1 IS A BETTER TARGET FOR ANTICANCER THERAPEUTICS?

Both the C and N termini of APE1 are involved in repair and redox functions of APE1 and are associated with cell growth and cancer promotion by two different independent mechanisms. Various endonuclease inhibitors and redox inhibitors have been designed to arrest DNA repair and the function of TFs by blocking the C- and N-terminal activities, respectively. However, the question remains of which APE1 domain to target to best sensitize cancer cells in anticancer therapy.

To target endonuclease activity, i.e., C-terminal DNA repair activity, inhibitors are designed as specific or nonspecific. Specific inhibitors are relatively novel compared to nonspecific inhibitors. They bind with the DNA repair-related C-terminus of APE1 and prevent it from interacting with DNA, preventing AP site repair.[131,161] These inhibitors have fewer off-target effects.[144]

Various 3D pharmacophore models were designed based on APE1 interactions with the abasic deoxyribose 3′- and 5′-phosphate backbone in the cocrystal structure of APE1 with the substrate AP DNA, as shown in Figure 13.[142] Many interactions that play a key role in determining the pharmacophoric requirements of APE1 inhibitors were observed. Arg177 was found to interact with the negatively charged 3′-phosphate of the AP DNA fragment. A hydrophobic pocket containing

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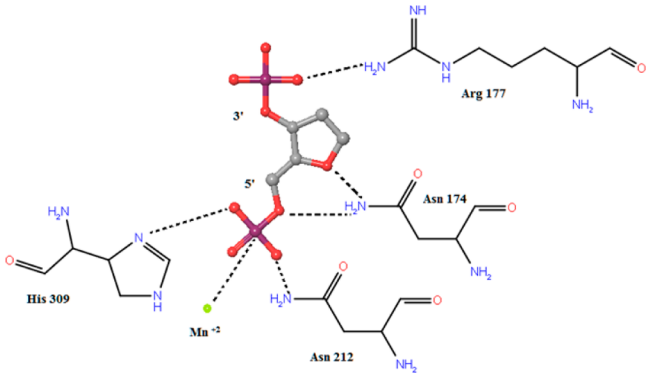

Figure 13. Interactions of residual amino acids of APE1 with the substrate AP DNA.

amino acids Phe253, Trp280, and Ile282 was found to bind the AP deoxyribose sugar moiety. H-bonding interactions of the 5′-phosphate were observed with residues Asn174, Asn212, and His309, and the Mn^{2+} metal ions.[142] On the basis of this information, three-dimensional pharmacophore models 1, 2, 3, and 4 in Figure 14 were designed.

Various endonuclease inhibitors proposed from these models had good IC_{50} values.[142] Model 1 (Figure 14) resembles AP DNA in which central hydrophobic (H) core corresponds to tetrahydrofuran (THF) that mimics natural deoxyribose sugar, and two negatively ionizable (NI) groups resemble its 3′- and 5′-phosphate groups. Model 2 consists of H-bond acceptor (A) feature resembling the oxygen atom of THF and two NI that represent the negatively charged 3′-phosphate of AP DNA. Model 3 includes three A (A1−A3) features and one NI group. A1 involves the interaction of the oxygen atom of THF with the side chain amine of Asn174. A2 and A3 represent interactions of APE1 amino acid residues Asn174 and His309 with the O5′ and O1P oxygen atoms of the 5′-phosphate. Model 4 does not contain any NI feature but consists of three A (A1−A3) features and one hydrophobic (H) feature. A1 represents the 3′-phosphate interaction with the side chain guanidine of Arg177. The H feature represents THF that mimics the natural abasic deoxyribose.[142] Therefore, these compounds were designed to be competitive inhibitors of the endonuclease activity of APE1.

Alternatively, nonspecific repair inhibitors have affinity for the AP site of DNA. Only one compound, methoxyamine, is reported to be a nonspecific inhibitor. This compound was identified by random in silico screening of a compound library. It was found by molecular modeling studies that methoxyamine does not bind the repair active site but is still a repair inhibitor. Further studies revealed that methoxyamine binds to the 3′-OH of the AP DNA and forms an irreversible adduct with the DNA, inhibiting APE1 from interacting with DNA for its repair function.[73] However, this compound was found not to be specific for AP DNA only. Methoxyamine has various additional cellular targets. Although it inhibits repair activity, it is not considered to be a successful repair inhibitor. The design and analysis of specific APE1 repair activity inhibitors are much further along than for methoxyamine.

As we have reviewed, redox inhibitors act by forming covalent bonds with the reduced active Cys65 residue, which is responsible for redox activity through the N-terminus of APE1. Redox inhibitors convert the reduced form of Cys65 in APE1 to the oxidized form, leading to Cys65 being unavailable for redox activity, further reduction/activation of TFs, enhanced DNA binding, as well as the transcription and expression of various genes involved in many cancer-enhancing signaling pathways.

Benzoquinone (e.g., E3330) and naphthoquinone analogs have been identified as general pharmacophores for redox inhibitors.[157] The sulfur of Cys65 attacks benzoquinone and naphthoquinone derivatives (Michael acceptors) in a Michael fashion (Figure 15).[139]

These Michael acceptors are specific not only for the sulfur of Cys65 but also to any nucleophile in the physiological system, resulting in additional off-target effects. E3330 has also been found to suppress the level of NF-κB by impairing its DNA-binding activity in a dose-dependent manner.[162] This could be due to Michael addition of the cysteine residue of NF-κB with E3330. These redox inhibitors can be described as oxidizing agents and are not specific to Cys65. However, they can oxidize other moieties as well and may disturb the redox balance in physiological systems. In this manner, redox inhibitors can act as cancer promoters by generating ROS, which may further induce abnormal cell signaling.

9. CONCLUSIONS

Finding novel targets to overcome resistance in chemotherapy is a continuing process in drug design and development. In this context, APE1/Ref-1 is a very interesting and valuable target

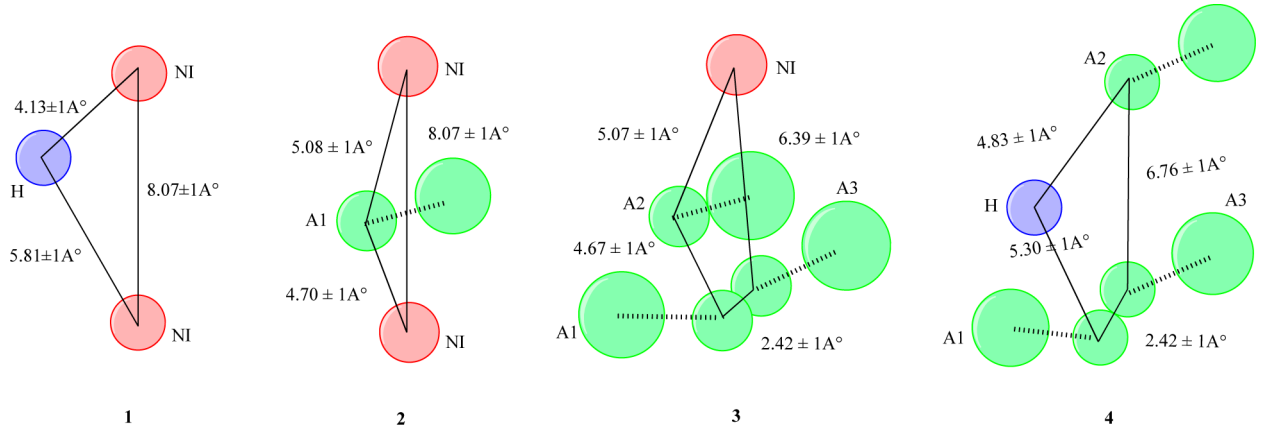

Figure 14. Pharmacophoric 3D models designed from interaction of the AP site DNA with APE1 repair active residues: NI, negatively ionizable group (red); H, hydrophobic moiety (blue); A, hydrogen bond acceptor (green).

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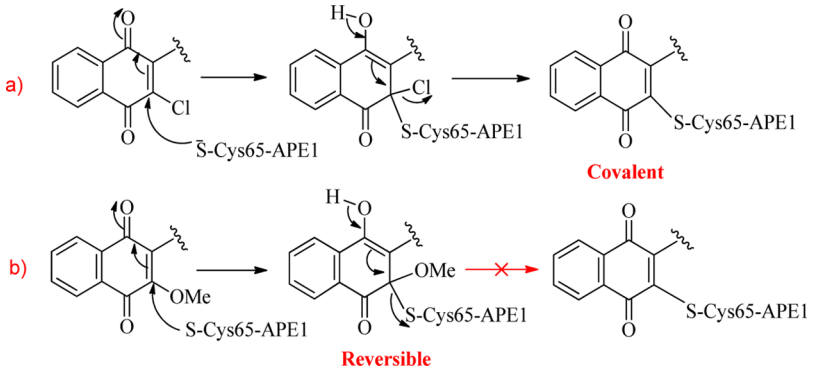

Figure 15. Redox inhibition mechanism of various analog of E3330: (a) irreversible and (b) reversible.

enzyme, with both domains (C and N termini) participating differently in the enhancement of cancer incidence and progression. Various APE1 inhibitors have been designed and developed to restrict chemoresistance. Little success has been achieved until now, and only two molecules, which were identified through random screening, have reached clinical trials. A recently described X-ray cocrystal structure of APE1 with the endogenous ligand has opened up the scope for the rational drug design of inhibitors using in silico approaches. Additionally, while comparing the inhibitors of both domains, we have found that specific inhibitors of the repair activity of APE1 are being pioneered and should be explored further. Redox inhibitors, on the other hand, can show additional off-target effects due to their mechanism(s). Additionally, redox inhibition nonselectively inhibits p53 and p21, which are tumor suppressor proteins and are activated by APE1, as described earlier. p53 and p21 play an important role in regulation of the cell cycle and cancer prevention. p53 has been described as "the guardian of the genome", and it directly controls p21.[163,164] A slight inhibition of their expression can transform a normal cell into a cancerous cell. Therefore, the design and synthesis of specific DNA repair APE1 inhibitors from pharmacophoric models that mimic AP DNA may provide a better way of addressing cancer resistance.

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All authors contributed to the writing of this manuscript. All of the authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AP-1, activator protein 1; APE1, apurinic/apyrimidinic endonuclease 1; ATA, aurin tricarboxylic acid; ATF, activating transcription factor; ATRA, all-trans-retinoic acid; BER, base excision repair; CREB, cAMP response element-binding protein; DSB, double strand break; EGR-1, early growth response protein 1; ETC, electron transport chain; GBM, glioblastoma multiforme; HCC, hepatocelluar carcinoma; HLF, hepatic leukemia factor; HR, homologous recombination; IR, ionizing radiation; LP, long patch; LOPAC, library of pharmacologically active compounds; MDR1, multidrug resistance; MGMT, O6-methylguanine-DNA methyltransferase; MMR, mismatch repair; MMS, methanesulfonate; mtDNA, mitochondrial DNA; MX, methoxyamine; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; NF-kB, nuclear factor κ light-chain enhancer of activated B cells; Pax 6, paired box protein; PTEN, phosphatase and tensin homolog; Polβ, polymerase β; Ref-1, redox effector factor 1; RNS, reactive nitrogen species; SP, short patch; SSB, single strand break; TF, transcription factor; TMZ, temozolomide; TRF2, telomeric repeat binding factor 2; TRX, thioredoxin

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