Review

Revisiting Two Decades of Research Focused on Targeting APE1 for Cancer Therapy: The Pros and Cons

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Abstract: APE1 is an essential endodeoxyribonuclease of the base excision repair pathway that maintains genome stability. It was identified as a pivotal factor favoring tumor progression and chemoresistance through the control of gene expression by a redox-based mechanism. APE1 is overexpressed and serum-secreted in different cancers, representing a prognostic and predictive factor, and a promising non-invasive biomarker. Strategies directly targeting APE1 funtions led to the identification of inhibitors showing a potential therapeutic value, which some of them are currently in clinical trials. Interestingly, evidence indicates novel roles of APE1 in RNA metabolism still not fully understood, including its activity in processing damaged RNA in chemoresistant phenotypes, regulating oncomiRs maturation, and oxidized-RNA decay. Recent data point out a control role of APE1 in the expression and sorting of oncomiRs, within secreted extracellular vesicles (EVs). This review is focused on giving a portrait of the pros and cons of the last two decades of research aiming at the identification of inhibitors of the redox or the DNA-repair functions of APE1 for the definition of novel targeted therapies for cancer. We will discuss the new perspectives in cancer therapy coming from the unexpected finding of the APE1 role in miRNA processing for personalized therapy.

Keywords: APE1; inhibitors; cancer

1. A brief introduction to APE1 biology and different functions

The acronym APE1/Ref1 (or more simply APE1) stands for apurinic/apyrimidinic (AP) endondeoxyribonuclease-reduction/oxidation 1, which is a well-known protein with multifunctional roles ranging from the endodeoxyribonuclease activity on DNA and RNA to the hub role in several reduction/oxidation (redox) signaling pathways [1–3].

Historically, APE1 has been largely known for its function during the base excision repair (BER) pathway [4], in which non-bulky DNA lesions are repaired. During the BER, APE1 works as AP-endonuclease, being able to cleave abasic sites that are generated spontaneously or by the action of specific glycosylases. The single strand break generated by APE1 cleavage is then brought to complete repair by other downstream BER enzymes (i.e. Polβ, XRCC1, FEN1, Ligase III). Another main cellular role of APE1 is working as a redox hub for several transcription factors (TFs). Indeed, the reduction of some TFs (i.e. NF-κB, p53, Hif-1α, AP-1, Pax-5/8, etc.) by APE1 allows their activation and consequently the initiation of the transcription of specific genes (i.e. IL-8, SIRT-1, VEGF, etc.). An additional role of APE1 in transcriptional regulation is due to its capacity to stabilize G-quadruplex (G4) which are stable conformational structures in the G-rich DNA portion of certain human promoters [5–7]. Although, following oxidative stress, the newly formed 8-oxoguanine (8-oxoG) can stall the transcription due to its destabilizing effect, there is evidence that its presence in some promoters induces the formation of BER-stabilized G4 that enhances gene expression [8,9]. In this context, the binding of APE1 to G4 sequences promotes G4 folding. Moreover, the acetylation of

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APE1 enhances its residence time on DNA and stabilizes G4 structures in cells [10]. In this way, APE1 facilitates transcription factor loading to the promoter modulating the gene expression [11]. Finally, attention will be paved to recently characterized APE1 functions, especially those involved in RNA metabolism [12–16]. Specifically, APE1 has resulted able to cleave abasic RNA [13] and damaged ribonucleotides embedded in DNA [15,16], discovering it as an efficient ribonuclease. Furthermore, only in the last years, concurrent with its novel and unsuspected involvement in RNA-metabolism, it has been described that APE1 can be present in subcellular condensates formed through liquid-liquid phase separation mechanisms (LLPS) [17–19] and can be secreted by tumoral cells through extracellular vesicles (EVs) [20].

These intriguing roles of APE1 have been discovered in three decades of constant interest in this protein, delineating them in physiological and pathological contexts and making this protein an attractive therapeutic target for several pathologies, including cancer [21]. Indeed, targeting APE1, after more than 20 years of attempts, still represents an important challenge in cancer therapy. In this review, we will focus on the dysregulation of APE1 in cancer and then we will describe known inhibitors of the principal functions of APE1, paving the way for novel functions involved in chemoresistance and potentially used as new therapeutic targets.

2. APE1 and cancer: a focus on polymorphisms and tissue expression

As previously discussed, being entailed in such focal cellular processes, APE1 dysregulation has a great impact on pathologies like cancer, making it an attractive therapeutical target [2,22]. APE1 dysregulation is involved, at three different levels, in tumour development, as it may concern alterations to its genetic sequence, to its expression, or to its localization [2].

Several studies pointed out the important role of single nucleotide polymorphisms (SNPs), present in APE1 gene, in cancer pathology [23] (Figure 1). The most common and studied APE1 variant is Asp148Glu (D148E), which is present in 48,5% of the population [24]. X-ray crystallography experiments showed that this variant lacks significant structural changes and its considered benign [25]. As a matter of fact, the protein bearing this SNP holds normal AP endonuclease and DNA binding properties, but its 3′-RNA phosphatase and endoribonuclease activities are somehow affected [14,26]. The role of this polymorphism in cancer is controversial, due to several conflicting data in literature [24]. This common variant has been widely studied since more than a hundred publications can be found regarding this topic. Numerous studies and meta-analysis associated D148E variant with an increased risk of different cancers, while others reported the opposite pattern even in the same tumour type [2,24,27]. Another APE1 polymorphic variant associated with cancer and biochemically studied is Arg237Cys (R237C) [28,29]. This substitution is associated with endometrial cancer [28,29] and it has greater consequences on the functional activity of the whole protein [25]. Indeed, the protein X-ray crystal structure revealed that this aminoacidic variation causes significant shifts in adjacent DNA binding residues, leading to a great diminution (~3-fold) of APE1 DNA binding ability [25]. Remarkably, this polymorphic variant has a reduced ability to associate with its BER partners, such as polymerase β (Pol β) and XRCC1 [23]. Indeed, the X-ray structure shows that also lysine 244 (K244) changes its position [25], which has been implicated in APE1-Pol β protein-protein interaction [30]. It has also been reported that R237C variant has a reduced AP-endonuclease activity [23], reduced 3′->5′ exonuclease and 3′-damage excision activities [29] and it has a reduced incision capacity close to nucleosomes [31]. One more endometrial tumour-associated variant is Pro112Leu (P112L) [28], which had comparable AP-endonuclease activity to the wild type form [29].

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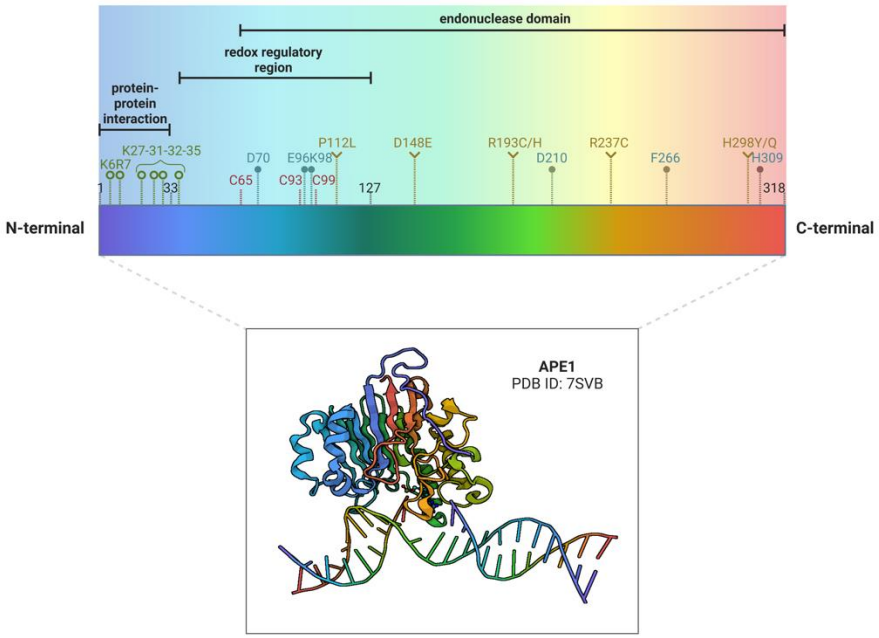

Figure 1. Human APE1 structure and key residues. Schematic representation of APE1 primary sequence, in which the most important aminoacids are highlighted. In the first 33 residues, required for protein-protein interaction, residues K6 and K7 (green, o) are involved in the shuttling between the nucleus and cytoplasm. Residues K27, K31, K32 and K35 can be acetylated and are essential for proteosomal cleavage. The redox regulatory region in included between aminoacid 35 to 125 in which the main residues involved are C65, C93, and C99 (red). The endonuclease domain spans between 65 to 318 (residues indicated in blue, •). Specifically, E96 is involved in divalent metal coordination, while D210 and H309 have functions in the hydrolytic reaction. Other important residues in the endonuclease domain are D70, which is implicated in the 3'-phosphodiesterase activity, K98, important for the nucleotide incision repair (NIR) and F266, which is involved in the 3' to 5'-exonuclease activity. Lastly, P112L, D148E, R193C/H, R237C, and H298Y/Q (yellow, o) are some of the polymorphisms of APE1 that will be discussed in this Review. Below, the tridimensional structure of APE1 is reported (PDB ID: 7SVB). Created with BioRender.com.

In this review, we performed an analysis on cBioPortal to find APEX1 SNPs or insertion/deletions (IN/DELs) related to different cancer types, considering a curated selection of non-redundant studies (213 studies selected, 69223 samples, 65853 patients) (https://bit.ly/3M9OYa7) [32,33]. The somatic mutation frequency of APEX1 was 0.2% with 108 unique variants. None of the variants detected was a driver mutation for cancer and most of them were sporadic. The most frequent variants were R193C/H, D148E, and H289Y/Q. Interestingly, even if R193C and R193H variants were detected to a comparable extent to D148E in the selected cohort of studies, there is no work in the literature that focuses on this mutation and on its functional impact. According to Mutation Assessor tool [34,35], theoretically, R193H has a low impact on the protein functional activity, while R193C might have a worse impact. There are no functional studies even about H289Y/Q variants, which are predicted to have a neutral impact on protein activity. In Table 1, we report every variant found with the cBioPortal tool for each tumour type.

Table 1. APE1 polymorphisms in different cancer types were detected with the cBioPortal tool.

Cancer	Polymorphisms
Adrenocortical carcinoma	D283H; X20_splice
Ampullary cancer	M271T
Bladder cancer	D15H; S146L; Q109E; E217*
Bone cancer	V131M

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Breast cancer	L244Tfs*8; Q51*; H289Y; G5A; K7Rfs*75; R185W; A60G
Cervical cancer	R281C; K7R
Colorectal cancer	A273T; R193C; R221H; G130D; R247Q; A230D; M271I; R221H; R181*; E242D; R274*; P293S; P49Qfs*33; L220I; T233M; N226Efs*26; V131M; L291Vfs*6; R221C; K63E; D210N; R281H; X82_splice; E101D; K77N; P331H; G306S; G132D
Endometrial cancer	R193H; S164L; R221H; A170V; P223L; V84I; V278D; R281H; K228T
Gastric cancer	R281C; P122A; K27N
Glioma	H289Q; Q245R; G80E; D142N
Head and Neck cancer	P48H
Leukaemia and Lymphoma	R181Q; L17P; K7R; R187H
Liver cancer	G5E; W280S; L220P; W280R; D50Rfs*28; G8R; H289Y, N226Efs*26; Y264_G279del
Lung cancer	G41C; V206Cfs*11; V142Sfs*8; E16Q; P331T; E149Q; X147_splice; R177*; D90H; Q51*;X237_splice; R193C; R28S; M271del; V206Cfs*11; S115F; G8R; I146V; D148E
Melanoma	G241W; E16K; G127V; R136S; P122T; D148E; A263V; K7Rfs*75; L108F; V69L
Oesophageal cancer	E46D; D251N; M270Nfs*14; F165V; N102I; L291Vfs*6; K3R; G145D
Ovarian cancer	Q95*, V168I; R193H; L291Vfs*6
Pancreatic cancer	R193C; M271del
Prostate cancer	P139Q; A30T; R187H
Renal cancer	E149*
Sarcoma	R187L; K35Rfs*11; K35Q
Skin cancer	P89S

APE1 overexpression and altered localization are prominent features of several different tumours and most of the time they have been associated with poor prognosis and malignant phenotypes. We further provide a description of how APE1 is altered in cancer and how this impacts tumorigenesis (Table 2).

Table 2. APE1 dysregulation in tumours: from its expression and distribution to its value as a diagnostic biomarker.

Cancer	Expression	Diagnostic value	Localization	Refs
Bladder cancer (BCa)	Protein overexpression, associated with poor survival and invasion.	Serum and urine levels as a diagnostic biomarker.	• In non-invasive, low-grade tumours: localization mainly in the nucleus. • In invasive, high-grade tumours: both nuclear and cytoplasmatic localization.	[36–42]
Breast cancer	• Conflicting data on protein expression: in some cases, overexpression is associated with malignant	*n.d.*	Nuclear localization.	[43–48]

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	phenotype and unfavourable prognosis; in other cases low APE1 is associated with aggressive triple negative phenotype; • Deregulation of acAPE1.			
Cervical cancer	High protein expression associated with lymph node metastasis, EMT and decreasing radiosensitivity.	*n.d.*	• Moderate and heterogeneous nuclear staining; • Radioresistant cervical cancer cell lines show higher levels of cytoplasmatic APE1 and lower levels of nuclear protein.	[49–52]
Colorectal cancer (CRC)	• Protein overexpression in cancer tissues, increasing from benign to malignant forms; • Overexpression of acetylated APE1 in tumour tissues, positively correlated with 5-FU resistance; • Presence of both the full length and the N-terminal truncated (NΔ33) proteins;	Serum APE1-autoantibodies levels as a diagnostic biomarker.	• Nuclear and cytoplasmatic localization; • The acetylated form accumulates in the nucleus.	[53–60]
Cutaneous Squamous Cell carcinoma (cSCC)	Protein overexpression in tumour tissues, which promotes proliferation of cancer cells and migration by EMT.	*n.d.*	*n.d.*	[61]
Gastric carcinoma	mRNA and protein upregulation, correlated with lymph node metastasis, depth of invasion and poor prognosis.	Serum levels as a diagnostic biomarker for metastasis prediction.	Nuclear and cytoplasmatic localization.	[62–65]
Glioma	• Conflicting data available: in some cases APE1 overexpression; in other cases low mRNA and protein expression associated with poor overall survival; • APE1 expression increases following treatment and recurrence.	*n.d.*	Nuclear localization.	[66–71]
Head and Neck Squamous Cell carcinoma (HNSCC)	• *In oral SCC (oSCC):* Protein overexpression, correlated with nodal status and lymph node invasion, shorter overall survival and disease free survival;. • *In laryngeal SCC (LSCC):* protein	*In oral SCC (oSCC):* Serum levels as a diagnostic biomarker, with high levels correlated with late TNM stages,	• *In oral SCC (oSCC):* mainly nuclear localization, with weak cytoplasmatic expression. • *In sinonasal SCC (sSCC) and SCC with*	[72–78]

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	overexpression in cancer tissues. • *In sinonasal SCC (sSCC) and SCC with inverted papilloma (SCCwIP):* protein overexpression in cancer tissues. • *In lip SCC (lSCC):* protein overexpression in cancer tissues.	lymph node metastasis and worse pathologic differentiation.	*inverted papilloma (SCCwIP):* vivid nuclear localization, associated with metastasis; higher cytoplasmatic staining in sSCC, associated with T-stage and histological grade. • *In lip SCC (lSCC):* nuclear localization.	
Liver cancer	• mRNA and protein overexpression, correlated with poor survival and cancer aggressiveness; • Presence of both the full full-length and the N-terminal truncated (NΔ33) proteins.	Serum levels as a diagnostic biomarker.	• Strong nuclear and cytoplasmatic positivity, with higher cytosol expression in poorly differentiated tumours; • In lower grade tumours, the cytoplasmatic positivity corresponds to mitochondrial accumulation.	[79–84]
Lung cancer	• mRNA and protein overexpression in NSCLC (non-small-cell lung cancer), which are associated with linfonodal metastasis and EMT promotion; • Increase of APE1 expression after cisplatin treatment; • High levels of APE1 acetylated form; • Presence of both the full length and the N-terminal truncated (NΔ33) proteins.	High post-treatment serum levels are associated with lower overall survival.	• Nuclear and cytoplasmatic staining: higher cytoplasmatic localization correlates with poor prognosis; • APE1 acetylated form is strictly nuclear.	[53,85–94]
Melanoma	mRNA and protein overexpression associated with vascular invasion, high mitotic rate, lower response to therapy and poor prognosis.	*n.d.*	Nuclear localization.	[95–98]
Oesophageal carcinoma (EAC)	Protein overexpression in tumour tissues, associated with worse overall survival.	*n.d.*	Mainly nuclear localization.	[99–104]
Osteosarcoma	• Protein overexpression in cancer samples, associated with poor prognosis and cisplatin resistance.	*n.d.*	• Mainly nuclear localization and variable cytoplasmatic staining; • High cytoplasmatic localization correlates with poor response to cisplatin therapy.00	[105–111]

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Ovarian cancer	Protein overexpression in tumour tissues, associated with advanced stages, platinum resistance and poor chemosensitivity, decreased overall survival and lymph node metastasis.	*n.d.*	• Strong nuclear and cytoplasmatic localization, heterogenous between different histological subtypes; • Cytoplasmatic localization increases from well to poorly differentiated tumours and it is higher in advanced stages; • Cytoplasmatic localization is associated with lower progression free survival time and decreased overall survival.	[99,112–119]
Pancreatic adenocarcinoma (PDAC)	• Protein overexpression in tumour tissues, associated with poor prognosis and tumour aggressiveness; • Elevated levels of acetylated APE1; • Presence of both the full full-length and the N-terminal truncated (NΔ33) proteins.	*n.d.*	Strong nuclear staining in tumour tissues, with cytosol staining only in advanced stages.	[53,85,99,120–122]
Prostate carcinoma (PCa)	Protein overexpression in cancer samples.	*n.d.*	Nuclear and cytoplasmatic localization.	[123,124]
Salivary gland carcinoma	• Protein overexpression in tumour tissues, increasing in dependence on malignant transformation and correlated to lymph node metastasis and invasive growth; • Higher protein levels in smaller tumours.	*n.d.*	Mainly nuclear staining, with nuclear and cytosolic staining in some malignant forms.	[125,126]

In bladder cancer (BCa), several studies detected high expression levels of APE1 protein in tumour tissues compared to normal adjacent tissues and they were associated with poor outcomes [37,39,42]. APE1 overexpression was also linked to lympho-vascular invasion features, as high VEGFA levels and infiltration of $CD163^+$ tumour associated macrophages (TAMs) [40]. Nuclei-cytosol distribution of APE1 was variable between high- and low-grade tumours: indeed, low grade cancers displayed increased APE1 levels only in the nucleus, while high grade invasive tumours showed increased positive staining even in the cytoplasm [37]. APE1 is also a promising diagnostic biomarker in BCa, as its levels in serum and urines were increased in contrast to normal healthy controls and they were associated with tumour grade and stage, recurrence, and invasion [36,38,41]. Interestingly, a study observed an increased secretory activity of D148E in bladder cancers, which contributed to increased serum levels of the protein in patients [127].

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Concerning hepatocellular carcinoma (HCC), APE1 is upregulated at both transcriptional and translational levels compared to normal liver tissues [79–81]. Moreover, the mRNA content increased with tumour progression and it is higher in less differentiated and more aggressive tumours [79]. Patients with greater APE1 protein levels exhibited unfavourable prognosis and lower overall survival [80,81]. Interestingly, both APE1 truncated forms, missing the first 33 residues (NΔ33 - 35 kDa) and APE1 full length (37 kDa) were detected in HCC tissue samples and HCC cell lines [82]. APE1 cellular distribution was altered in HCC: while APE1 staining was only nuclear in normal liver tissues, in tumour tissues there was also a significant fraction of the protein in the cytoplasm [83]. Cytoplasmatic APE1 was about three times higher in poorly differentiated tumours and it was associated with reduced overall survival [83]. Noteworthy, the cytoplasmatic staining was associated with APE1 mitochondrial accumulation in grade 1 and grade 2 HCC tumours, while in grade 3 tumours it was not [84]. Even in HCC, APE1 serum levels can be used as a novel diagnostic biomarker [82].

APE1 protein resulted overexpressed also in pancreatic adenocarcinoma (PDAC) tissues and cell lines and it was associated with tumour aggressiveness and poor survival [120–122]. The proteolytic form of APE1 NΔ33 has been detected even in PDAC tissues, with different abundance versus adjacent non-tumour tissue [53]. Interestingly, the acetylated form of APE1 (acAPE1) was overexpressed in PDAC tumours, while it was almost undetectable in healthy pancreatic tissues [53,85]. acAPE1 holds an increased AP-endonuclease activity, which has been proposed as a cancer mechanism to overcome chemotherapy genotoxic stress and uphold proliferation [85]. APE1 localization in PDAC was mainly nuclear and it was similar between primary tumours and their metastasis [121]. Cytosolic localization was observed only in tumour advanced stages and never in the absence of nuclear localization, while its complete absence was associated with invasion and poor differentiation [99,121].

Concerning prostate cancer (PCa), APE1 protein levels were upregulated compared to normal tissue and benign hypertrophy (BPH) [123,124]. Moreover, higher APE1 levels were observed in tumours bearing TMPRSS2:ERG fusion [124]. APE1 localization was only nuclear in normal prostate tissue and non-cancerous prostate cell lines, while there was an increase in the cytoplasmatic fraction in tumour tissues and tumoral cell lines [123].

APE1 overexpression occurs also in oesophageal carcinomas, like oesophageal adenocarcinoma (EAC) [100–102] and oesophageal squamous cell carcinoma (ESCC) [103,104], probably as a mechanism adopted by cancer cells to survive the genotoxic effects of bile reflux [103,104]. APE1 localization was mainly nuclear and it was also associated with worse overall survival in patients receiving platinum chemotherapy [99].

An *in silico* analysis identified APE1 as a central hub gene for gastric cancer, as its overexpression had a great prognostic value in two analysed datasets (GSE1611533, GSE54129) [62]. Indeed, APE1 was overexpressed at both transcriptional and protein level in gastric cancer [63,64]. APE1 staining was weak in normal non-cancerous gastric tissues, while it was both nuclear and cytoplasmatic in tumour tissues [64]. High levels of APE1 were also correlated with invasion and poor prognosis [64], as its serum levels are a valuable diagnostic biomarker for lymph node metastases prediction [65].

APE1 was also upregulated in salivary gland carcinomas and its levels increased depending on malignant transformation of the tumour [125]. APE1 overexpression was higher in smaller tumours displaying lymph node metastasis and invasive growth [125,126]. APE1 localization was mainly nuclear in every salivary gland tumour subtype, except for adenoid cystic carcinomas in which it showed both nuclear and cytoplasmatic localization [125,126].

Furthermore, the overexpression of APE1 protein and mRNA levels was reported also in non-small-cell lung cancers (NSCLCs) [86–89]. High APE1 expression was associated with poor prognosis, invasion, and chemoresistance, as its levels increased after treatment with platinum compounds [88]. Moreover, high *post* treatment APE1 serum levels were correlated with poorer overall survival [90]. Nuclear APE1 staining was associated with favourable patient outcomes [91], while a higher cytoplasmatic localization correlated with poor survival and shorter relapse-free survival (RFS) [92–94]. Both full-length and truncated forms were found in lung cancer and interestingly APE1 was

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prevalently truncated at the N-terminus in NSCLC adjacent non-tumour tissues [53]. AcAPE1 was overexpressed in NSCLC tumours, with a strictly nuclear localization [53,85].

Several studies identified overexpression of APE1 protein in ovarian cancers, which has been associated with advanced tumour stages and decreased overall survival [99,112,113]. Moreover, patients with high levels of APE1 showed more frequent resistance to platinum therapy [99,112,114]. The protein-protein interaction between APE1 and nucleophosmin 1 (NPM1) has been extensively examined in ovarian cancer, as the levels of the two proteins were positively correlated and associated with tumour aggressiveness, malignant phenotype, lymph node metastasis and poor chemosensitivity [112,115]. It has been shown that compounds which impair this interaction can add a synergistic effect to traditional chemotherapeutic molecules [116] APE1 localization seemed to be heterogenous in ovarian cancers, depending on stage and histological subtype [22]. Some studies showed prominent cytoplasmatic staining, which increased from well to poorly differentiated cancers and it was higher in advanced stages tumours [114,115,117,118]. In non-responding to cisplatin cases, the observed APE1 overexpression was mainly at cytoplasmatic level, a feature that was observed also in cisplatin resistant cell-lines [114]. Interestingly, almost 90% of patients with abnormal levels of cytosolic APE1 displayed an abnormal distribution of NPM1 [115]. Additionally, cytosolic APE1 can be considered an independent predictive factor for poor progression free survival and for poor overall survival in ovarian cancers [117]. Other works showed prominent nuclear APE1 staining, which increased during tumorigenesis and was associated with survival time [113,119]. Other studies showed an increase of APE1 in both compartments but with higher nuclear staining, which was associated with cancer aggressiveness, lower debulking after surgery, platinum resistance and lower overall survival [99,114].

For what concerns breast cancer, different studies reported conflicting results about APE1 protein expression. Some works described APE1 overexpression in breast cancers, which was mostly nuclear and correlated with malignant phenotypes and unfavourable prognosis [43–45]. Contrary to the pattern of increased acetylation observed in other cancer types [53,85], APE1 acetylation resulted in heterogeneous and deregulated in breast cancer [46]. Even in this case, the functional interaction between APE1 and NPM1 in promoting platinum resistance has been described [47]. In contrast to these findings, another study showed that lower levels of APE1 were associated with tumour aggressiveness and triple negative phenotype [48]. Interestingly, another study showed that, within the Ki-67 low-level expression group, lower levels of APE1 were associated with poor overall survival [44].

APE1 protein levels were upregulated even in cervical tumours and they were associated with Epithelial-to-Mesenchymal transition (EMT), lymph node metastasis and poor radiosensitivity [49–52]. APE1 localization was widely heterogeneous between cervical tumours, showing mainly nuclear staining [50]. Remarkably, there was a significant difference in subcellular localization of APE1 between radiotherapy non-responding and responding tumour cell lines: indeed, radioresistant cervical tumours cell lines showed higher levels of the cytoplasmatic fraction and lower levels of the nuclear protein, suggesting a role of cytosolic APE1 in radio-resistance promotion [51].

Several studies described an overexpression of APE1 in colorectal cancers (CRC), observing also a gradual increase of its expression during tumour progression [54–57]. APE1 levels resulted high also in liver metastasis of CRC [58]. APE1 localization in CRC was both nuclear and cytoplasmatic, with most cells displaying mixed or exclusive cytoplasmatic localization [54]. Even in CRC tumour samples and cell lines, nuclear acAPE1 was overexpressed [53,59] and it positively correlated with resistance to 5-Fluorouracil (5-FU) [59]. Moreover, both full-length and truncated forms were detected in colon cancer [53]. Interestingly, the levels of serum APE1 autoantibodies are valuable diagnostic biomarkers for CRC [60].

Regarding glioma, conflicting data are available. Some studies described an overexpression of APE1 in tumoral tissues compared to healthy ones [66,67], with a 13-fold increase of AP-endonuclease activity in 93% of tumours [66]. Glioma radioresistant cell lines displayed higher levels of APE1 compared to responding cell lines [68]. Indeed, an increase in APE1 expression was observed in patients after treatment and recurrence [69]. On the other hand, different studies evidenced low

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mRNA and protein expression in adult high-grade gliomas, associated with poor overall survival [70,71]. Moreover, APE1 localization in gliomas was predominantly nuclear [70].

For what concerns melanoma, several studies identified APE1 overexpression at both transcriptional and translational levels [95–97]. Indeed, APE1 was overexpressed in melanoma cancer cell lines and in clinical samples, showing a prominent nuclear localization in both cases [96,97]. High mRNA levels were associated with vascular invasion, high proliferation rates, poor relapse free survival, and overall survival [95,98]. Patients with higher levels of APE1 also showed a lower response to therapy [98].

APE1 was overexpressed also in another skin tumour, namely cutaneous squamous cell carcinoma (cSCC) [61]. APE1 upregulation in cSCC was associated with increased proliferation and migration by EMT [61]. APE1 was dysregulated in several Head and Neck squamous cell carcinomas (HNSCC). In oral SCC (oSCC), APE1 was overexpressed at protein level and its high expression was significantly correlated with nodal status, shorter overall survival and disease-free survival [72,73]. APE1 localization in oSCC was mainly nuclear, but a translocation to cytoplasm was observed after cisplatin treatment [72,74]. In oSCC, APE1 serum levels are a promising diagnostic biomarker [75]. Indeed, high levels of serum APE1 (sAPE1) are associated with late TNM stages, lymph node metastasis and worse pathological differentiation [75]. Patients with lower levels of sAPE1 went through longer disease-free survival after post-surgery cisplatin therapy and longer overall survival [75]. APE1 overexpression was observed also in laryngeal SCC (LSCC) [76]. APE1 levels resulted upregulated even in sinonasal SCC (sSCC) and SCC with inverted papilloma (SCCwIP), with a vivid nuclear localization associated with metastatization [77]. sSCC tumours showed higher cytoplasmatic staining compared to SCCwIP [77]. Moreover, exclusive cytoplasmatic staining was associated with higher T-stage and histological grade [77]. Lastly, APE1 overexpression characterised also lip SCC (lSCC), with a strong nuclear localization [78].

Furthermore, APE1 levels were upregulated in osteosarcoma and they were associated with poor prognosis and cisplatin resistance [105–111]. APE1 localization was both nuclear and cytoplasmatic [105,110]. Patients with higher levels of the protein in the cytoplasmatic fraction were less responding to cisplatin treatment and experienced recurrence and metastasis [105].

Therefore, in general, APE1 is significantly overexpressed in different kinds of cancers and subcellular distribution may significantly change depending on the specific tissue and tumoral stage.

3. APE1 as a promising therapeutic target

In the last decades, APE1 has emerged as a promising therapeutic target in cancer, either for its role in DNA repair or for its redox activities. In the next paragraphs, we will dive deeper into these functions of APE1, highlighting the study progression around the discovery of specific inhibitors, principally employed in chemotherapy (Table 3). Last but not least, we will discuss the new roles of APE1 in RNA metabolism and in cell signaling through its secretion, hypothesizing these novel functions as new promising targets in cancer therapy.

Table 3. List of the principal APE1 inhibitors. The principal APE1 inhibitors are grouped for their APE1 function inhibited including the endonuclease activity, the redox activity, and the protein-protein interaction. For each inhibitor, the IUPAC name, the PubChem CID, the molecular formula and weight, and the structure are reported. In the end, the main references for each inhibitor are indicated. For more detailed information, refer to the text.

APE1 function inhibited	Name	IUPAC name	PubChem CID	Molecular Formula	Molecular Weight (g/mol)	Structure	Refs
Endonuclease	**Methoxyamine**	O-methylhydroxylamine	4113	CH_5NO	47.057		[137–140]

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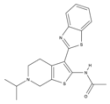
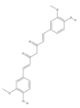
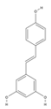
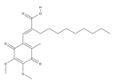
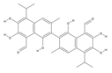
	Lucanthone	1-[2-(diethylamino)ethylamino]-4-methylthioxanthen-9-one	10180	$C_{20}H_{24}N_2OS$	340.5		[141–143]
	CRT0044867	7-Nitroindole-2-carboxylic acid	81409	$C_9H_6N_2O_4$	206.15		[21,144–146]
	Myricetin	3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one	5281672	$C_{15}H_{10}O_8$	318.23		[145]
	AR03	2,4,9-Trimethylbenzo[b][1,8]naphthyridin-5-amine	698490	$C_{15}H_{15}N_3$	237.30		[149]
	APE Inhibitor III	N-[3-(1,3-benzothiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl]acetamide	3581333	$C_{19}H_{21}N_3OS_2$	371.5		[153,154]
Redox	**Curcumin**	(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione	969516	$C_{21}H_{20}O_6$	368.4		[173,174]
	Resveratrol	5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol	445154	$C_{14}H_{12}O_3$	228.24		[97,175]
	APX3330	(2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl)methylidene]undecanoic acid	6439397	$C_{21}H_{30}O_6$	378.5		[22,177–187]
	APX2009	(2E)-N,N-diethyl-2-[(3-methoxy-1,4-dioxonaphthalen-2-yl)methylidene]pentanamide	71618575	$C_{21}H_{25}NO_4$	355.4		[177]
Endonuclease Redox	**Gossypol**	7-(8-formyl-1,6,7-trihydroxy-3-methyl-5-propan-2-ylnaphthalen-2-yl)-2,3,8-trihydroxy-6-methyl-4-propan-2-ylnaphthalene-1-carbaldehyde	3503	$C_{30}H_{30}O_8$	518.6		[190,191]

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protein-protein interaction	**Fiduxosin**	5-[4-[(3aR,9bR)-9-methoxy-3,3a,4,9b-tetrahydro-1H-chromeno[3,4-c]pyrrol-2-yl]butyl]-12-phenyl-8-thia-3,5,10,13-tetrazatricyclo[7.4.0.02,7]trideca-1(13),2(7),9,11-tetraene-4,6-dione	172307	$C_{30}H_{29}N_5O_4S$	555.6		[47,116,200]
	Spiclomazine	8-[3-(2-chlorophenothiazin-10-yl)propyl]-1-thia-4,8-diazaspiro[4.5]decan-3-one	65714	$C_{22}H_{24}ClN_3OS_2$	446.0		[47,116,200]
	SB 206553	1-methyl-N-pyridin-3-yl-6,7-dihydropyrrolo[2,3-f]indole-5-carboxamide	5163	$C_{17}H_{16}N_4O$	292.33		[47,116,200]

3.1. Targeting the APE1 endonuclease activity

The endonuclease function of APE1 is essential for its activity in the BER pathway. This role of APE1 depends mainly on its C-terminal region: the three most important residues involved in this activity are E96, which is implied in the coordination of divalent metals, D210 and H309, that are required for the hydrolysis reaction [128] (Figure 1). Other important residues that mediate different functions can be found in the C-terminal region: indeed, D70 is involved in the 3′-phosphodiesterase activity [129], K98 is required in the Nucleotide Incision Repair (NIR) [130] and F266 is implicated in the 3′ to 5′-exonuclease activity [131].

Previous studies identified different compounds that inhibit endonuclease APE1 activity, *in vitro* and in human cells, as summarized in different reviews [132–135]. Various groups, over the years, worked extensively towards the identification of potent small-molecule inhibitors able to target the DNA repair function of APE1, in combination studies, with the rationale that the blockade of APE1 endonuclease activity might have various therapeutic applications, particularly in cancer treatment, by sensitizing cancer cells to DNA-damaging agents and leading to tumor cell death. Although many studies support the inhibition of APE1 as a means of complementing current chemotherapeutic regimens and, accordingly, various chemical inhibitors have been developed, to date, a clinical candidate has yet to be realized. Indeed, despite their high *in vitro* activity, the toxicity and the selectivity of the majority of the reported endonuclease inhibitors remain to be established. In this section, we attempt to present the major APE1 inhibitors identified thus far and discuss their activity. It is not our scope to revisit all the inhibitors in depth; a comprehensive list of APE1 endonuclease inhibitors is reviewed in [128,135,136]. The published approaches utilized for the development of APE1 endonuclease inhibitors can be mainly categorized into (i) screening of commercially available compounds that were synthesized for targeting other molecules; (ii) computational screening, and (iii) pharmacophore modeling. One of the first studied molecules impairing APE1 repair activity was Methoxyamine (MX), an alkoxyamine derivative that reacts to form an imine with the aldehyde group in the ring-open form of the abasic lesion thereby indirectly blocking APE1 endonuclease activity [137,138]. Since MX was demonstrated to enhance the cytotoxicity effect of alkylating agents such as temozolomide (TMZ) in a wide variety of cancer cell lines both *in vitro* and in xenografts models [139,140], it advanced to clinical trials; however, to date, clinical studies have not shown success.

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A second compound that was first identified as a radiosensitizer of HeLa cells [141] and subsequently reported to be an inhibitor of APE1 by Luo and Kelly in 2004 [142], is Lucanthone or Miracil D. Lucanthone was shown to enhance the cell-killing effect of TMZ and an alkylating agent such as methyl methanesulfonate (MMS) in culture and was further characterized by Naidu and colleagues to bind to the hydrophobic pocket site of APE1 [143]. No other studies were reported successively but its specificity is still debated because part of its inhibitory effect is mediated by its ability to intercalate into DNA and through the inhibition of topoisomerase II and possibly other cellular proteins [21].

Afterward, numerous laboratories relied on high throughput screens (HTS) mainly based on fluorescence assay to identify inhibitors of APE1 endonuclease activity. In general, the identification of the potential hits was followed by different assays aiming to prove specificity and selectivity for APE1 inhibition including AP site incision assay, the ability of the compound to bind DNA *per se*, and the ability to enhance the cytotoxicity of alkylating agents (i.e. TMZ, MMS). It is worth mentioning that the inhibitors reported so far showed affinities in the μM range, that are not compatible with a suitable pharmaceutical agent and more importantly, none of them have been demonstrated to have utility in pre-clinical animal cancer models. CRT0044876 (7-nitroindole- 2-carboxylic acid) is the first biochemical and biological reported APE1 inhibitor identified through a fluorescein/dabcyl-based AP site incision assay [144]. Madhusudan and colleagues identified the compound CRT0044876 from a screen of a collection of structurally-diverse small molecules. Despite the initial promising results obtained in the potentiation of the cell-killing effects of MMS and TMZ, the reproducibility of this compound has been brought into question [21,145] and because of its poor solubility and permeability, this compound was further neglected until now where its usage has been proposed conjugated with platinum [146]. Considering the weak results obtained with CRT0044876, other screenings were then performed. Using a similar HTS approach, Seiple and colleagues screened the 2000-compound NCI Diversity Set of small molecules and identified aromatic nitroso, carboxylate, sulfonamide, and arylstibonic acid compounds with μM affinities for APE1 protein [147]. Again, also for these compounds, the relatively high inhibitory potency observed *in vitro* did not find a parallel significant effect in cells. Successively, various one-off studies did not progress to lead optimization. For example, in 2009, prompted by the evidence that APE1 represents an attractive therapeutic target in anticancer drug development, Zawahir and colleagues utilized a pharmacophore-based approach that was used to carry out a virtual screen of a 365,000 small molecule library [148]. The known interactions of APE1 with AP site-containing DNA including components of hydrophobicity, H- bond acceptor, and negatively ionizable features were utilized to design a virtual screen. In the same year, Simeonov and colleagues employing a quantitative HTS screened the commercially available Library of Pharmacologically Active Compounds (LOPAC), identifying 6-hydroxy-DL- DOPA, Reactive blue 2, and myricetin as APE1 possible inhibitors [145]. Although these approaches predicted several molecules, they were not all tested in cell-based assays and thus have not been evaluated for cell permeability.

Successively, Kelley's group has also used a fluorescence-based high-throughput assay to screen a library of 60,000 small molecule compounds for the ability to inhibit APE1 AP endonuclease activity [149]. The most promising compounds were designated as APE1 Repair Inhibitor AR01, 02, 03, and 06. AR03 is chemically distinct from the previously reported small molecule inhibitors of APE1. This compound was demonstrated to inhibit the cleavage of AP sites *in vitro* of whole cell extracts and to potentiate the cytotoxicity of TMZ and MMS of glioblastoma SF767 cells. While it is cell-permeable, its planar fused-ring structure may suggest its DNA intercalating ability thus potentially being aspecific.

In 2011, Mohammed and colleagues focused on developing APE1 inhibitors for melanoma and glioma treatment using a structure-based drug design approach [96]. The crystal structure of APE1 was utilized to create four pharmacophore models, including the interactions of the previously identified inhibitor CRT0044876 with active site residues and molecular scaffolds designed to fit the ligand binding site. From the screening of 1679 hits, the authors identified compound 4 (N-(4-fluorophenyl)-2-(4-phenylsulfonyl-2- (p-tolyl)oxazol-5-yl) sulfanyl-acetamide) as the one with the

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highest AP endonuclease inhibitory activity and the potential to sensitize the activity of MMS and TMZ in both glioma and melanoma cell lines but not in HUVEC cells, suggesting specificity for malignant tissue.

In 2012, a new class of inhibitors of the catalytic endonuclease function was identified by Aiello et al. [150]. Compounds 32-35, which have a 3-benzylcarbamoyl-2-methoxybenzoic acid structure, showed the most active and selective inhibition activity of APE1. These compounds have the potential to be used in combination therapy with 5-fluorodeoxyuridine for colon cancer treatment. In the same year, using a docking-based virtual screening, 15 potential compounds were identified as inhibitors of the APE1 from a library of over 4 million molecules [151]. Two of these compounds, 36 and 37, were found to be potent inhibitors of the protein and could increase the toxicity of MMS. Through molecular dynamics simulations, it was discovered that these compounds may interact with the protein through important binding modes such as hydrogen bonds with specific residues and hydrophobic interactions by virtue of their quinoxaline core. Also in 2012, Simeonov's group performed a fully-automated HTS using a kinetic fluorescence assay on the NIH Molecular Libraries Small Molecule Repository and other collections, examining each agent at different concentrations [152]. They identified active APE1 inhibitors able to potentiate the genotoxic effect of MMS, leading to an increase in AP sites. The chemical structures of the most effective inhibitors namely MLS001196838, MLS000587064, MLS000737267, MLS000090966, and MLS000863573 would have served as starting points for medicinal chemists to further optimize them.

Another fluorescence-based quantitative HTS of 352 489 small molecules from the NIH Molecular Libraries Small Molecule Repository was performed by Rai and colleagues [153]. APE Inhibitor III (N-(3-(1,3-Benzo[d]thiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl)acetamide) has been demonstrated to potentiate MMS and TMZ activity in HeLa cells. This compound was further used and distributed by the vendors as one of the most promising APE1 inhibitors, however, also this compound has not significantly advanced beyond *in vitro* studies. Srinivasan et al., using the crystal structure of APE1, computationally constructed molecules that would sterically block its endonuclease site and identified molecules that all contain 2-methyl-4-amino-6,7-dioxoloquinoline structure [154]. The mechanism of action of the compounds was probed by fluorescence and competition studies in T98G glioma cell lines, which indicated for compounds 1 and 4 direct interaction between the inhibitor and the active site of APE1 protein.

In 2015, a pharmacophore model for APE1 small-molecule inhibitors was used to identify new compounds by the means of in silico screening of 10 159 compounds [155]. The virtual docking assay identify four compounds with a 2-methyl-4-amino-6,7-dioxoloquinoline core (AJAY 1-4); AJAY 4 showed the best results in the inhibition of cell growth, however, none of the compounds have advanced in clinical studies.

Another *in silico* novel approach was pursued by Trilles et al., who guided by X-ray crystal structures of APE1 and computational docking of solvents identify binding hotspots for small organic molecules [156]. Accordingly, they screened a library of macrocycles for inhibiting APE1 endonuclease activity and identified four novel macrocycles that they used as a starting point for designing APE1 ligands. From the initial screening of 66 compounds, four exhibited concentration-dependent inhibition of APE1 endonuclease activity (MC043, MC047, MC042, and MC019). Building on these hits, additional macrocycles were synthesized and macrocyclic lactams 13, 21, 24 have been demonstrated to be more effective in inhibiting APE1 endonuclease function in combination with MMS.

Unfortunately, none of the compounds developed so far have advanced to significant *in vivo* studies or clinical trials. Interestingly, very recently, two works argued about the specificity of some of the most prominent compounds that are usually sold by vendors as APE1 inhibitors. In the work of Pidugu et al., it has been demonstrated through structural, biophysical, and biochemical approaches that several reported small molecules are weak APE1 inhibitors [157]. In particular, through NMR chemical shift perturbation assay, they showed that CRT0044876 and three similar indole-2-carboxylic acids compounds (5-fluoroindole-2-carboxylic acid [96], 5-nitroindole-2-carboxylic acid and 6-bromoindole-2-carboxylic acid) bind at a pocket of APE1 that is distal from its

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active site. Furthermore, using Dynamic light scattering (DLS), they also demonstrated that CRT0044876 [144], myricetin [145], and APE Inhibitor III [153] form colloidal aggregates that could sequester APE1 causing non-specific inhibition. For this latter compound, also Xue and Demple recently raised the question about the specificity of this molecule [136]. Since APE1 Knockout lines (CH12F3 [158]) showed equal sensitivity to direct killing by APE Inhibitor III, being even more sensitive to APE Inhibitor III than its wildtype counterpart, the authors claimed possible off-target effects that must be taken into account when using these inhibitors at high dosages.

3.2. Targeting the APE1 redox activity

Unlike the BER function, which is highly conserved from prokaryotes (*E. coli* exonuclease III) to humans, the redox function is probably unique to mammalians [159]. Whereas the C-terminal of APE1 is mainly involved in the regulation of the endodeoxyribonuclease activity, the N-terminal, unstructured region is strongly implicated in the protein-protein interactions (PPI) and in the activation of several transcription factors (TFs) via a redox mechanism. The redox function of APE1 is exploited by cysteine residues sited at positions 65, 93, and 99 of the unstructured N- terminal region (Figure 1). These residues, are involved in the redox cycle responsible for controlling the reduced state of several TFs [159]. By reducing the TFs , APE1 makes them able to bind DNA. APE1 then returns to its basal state through another reduction, that occurs *via* a thiol/sulfide exchange with thioredoxin. Among the several TFs regulated by APE1, we include the principal such as NF B [160], AP-1 [160], HIF1 [161], STAT3 [162,163], p53 [164], NRF2 [165], Pax-5 and -8 [166] and others [167]. Given the roles of all these TFs in the cellular biological processes, the effects of APE1 as a redox signaling factor regards principally the promotion of the growth, migration, DDR signaling, and survival in tumor cells as well as inflammation and angiogenesis in the tumor microenvironment. Thus, the inhibition of APE1 redox activity can be a target for slowing growth and progression during tumoral processes. The pharmacological inhibition of APE1 redox activity causes a decrease in the ability of the transcription factors to bind to DNA [167–169] and thereby increases the cancer cells' response to chemotherapeutic agents [170,171].

Differently from the AP-endonuclease inhibitors, testing redox inhibitors resulted in more complications during these years, in part due to the arduous modalities of detection of the redox activity of APE1. In this paragraph, we propose a roundup of literature on a few redox inhibitors that emerged onto the scientific scene.

Dietary agents and several compounds from natural sources, such as soy isoflavones, resveratrol, and curcumin but also the vitamins ascorbate and α-tocopherol [172], were initially tested. Curcumin is a polyphenol with the potential for treatment or prevention of particular human diseases such as oxidative and inflammatory conditions, metabolic syndrome, arthritis, anxiety, hyperlipidemia, and cancers [173]. In 2017, it was demonstrated that Curcumin affects the APE1 redox function inhibiting the transcriptional activity of APE1 on AP-1 and NF- B genes *in vitro* [174]. For its multiple anti-inflammatories, anti-oxidant and anti-neoplastic properties, Curcumin was enrolled in more than 300 clinical trials. Resveratrol is a naturally occurring polyphenolic compound present in red wine and grapes. It has been demonstrated that it exhibits a neuroprotective role in models of central nervous system diseases, including cerebral ischemia/reperfusion injury [175]. By inhibiting APE1 redox function, resveratrol causes a significantly diminished activity of AP-1 and NF- B proteins in different human cancers models, enhancing the cytotoxicity of chemotherapy [97]. Similarly, utilizing soy isoflavones to block the redox signaling through APE1 and NF-κB dramatically increased prostate cancer cells' sensitivity to radiation [176].

About ten years ago, the Kelley' group synthesized a molecule that turned out to be highly promising in the inhibition of APE1 redox activity in several cancer models [22,177]. This molecule [(2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene]-undecanoic Acid, commonly denoted as APX3330 (or E3330) is a quinone derivative. Several studies were then performed by using different pathological models both *in vitro* and *in vivo* in which it was demonstrated that APX3330 selectively inhibits NF-κB-mediated gene expression through APE1 binding [178]. In 2009, Zou and colleagues demonstrated that APX3330 blocked the *in vitro* growth

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of pancreatic cancer-associated endothelial cells (PCECs) and EPCs, and the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into CD31(+) endothelial progeny. Specifically, the effect was attributable to a reduction of H-Ras expression and intracellular nitric oxide (NO) levels, as well as decreased DNA-binding activity of HIF-1 . Inhibition of the APE1 redox function by APX3330 might be a potent therapeutic strategy in solid tumors [179]. Indeed, APX3330 showed anticancer properties in pancreatic cancer, including inhibition of cancer cell growth and migration in several cancer cell lines and xenograft models in mice [180]. APX3330 inhibited the proliferation, migration, and tube formation of retinal vascular endothelial cells *in vitro* and reduced retinal angiomatous proliferation neovascularization *in vivo* [181]. As anticipated in the previous paragraphs, elevated expression levels of APE1 have been correlated with more aggressive phenotypes and poor prognosis of NSCLC. Recently, Manguinhas and colleagues demonstrated that APX3330, in combination with cisplatin, reduces H1975 cell viability, migration, and invasion highlighting its use as a boost of cisplatin in NSCLC cells [182]. Moreover, the inhibition of APE1 redox function through APX3330 combined with docetaxel treatment decreased the proliferative rate and cell migration and invasion of breast cancer cells MDA-MB-231 [183]. The APX3330 inhibitory activity was also assessed in pathological angiogenesis, such as retinal neovascularization [184]. Li and colleagues demonstrated that APX3330 treatment suppresses experimental choroidal neovascularization (CNV) *in vitro* and *in vivo*, demonstrating that APE1 regulates multiple transcription factors and inflammatory molecules, and is essential for CEC angiogenesis. APE1 is a novel candidate for therapeutically targeting neovascular eye diseases and alleviating the burden associated with anti-VEGF intravitreal injections [185]. Recently, it has been also demonstrated that APX3330 has the potential to be used for the treatment of γ-herpesvirus infection and associated diseases [186].

Given its promising and potential anti-angiogenic and antineoplastic activities obtained *in vitro*, in 2017, APX3330 has been enrolled in the APX_CLN_0011 Phase 1 clinical trial. This trial (ClinicalTrials.gov Identifier: NCT03375086) was a multi-center, open-label, dose-escalation oncology study of APX3330 in patients with advanced solid tumors. The study was completed in 2020 showing the assessment of APX3330 safety, anti-tumor activity, pharmacokinetic and pharmacodynamic profile [187], and the recommendations of Phase 2 study dose. Oral APX3330 demonstrated a favorable safety and tolerability profile and was suitable for Phase 2. In 2020, APX3330 has been enrolled in ZETA-1 Phase 2 clinical trial to evaluate its safety and efficacy to treat diabetic retinopathy (DR) and diabetic macular edema (DME). The trial was completed this year (2023) (ClinicalTrials.gov Identifier: NCT03375086). Oral administration of APX3330 and placebo have demonstrated a favorable ophthalmic and systemic safety and tolerability profile. Additional safety data from the ongoing ZETA-1 trial will be evaluated to further characterize the efficacy and safety of APX3330 for the oral treatment of diabetic eye diseases.

On the wave of the results obtained with APX3330, new analogues of this inhibitor were synthesized including RN8-51, 10-52, and 7-60. Data shown in [188] have demonstrated that the analogue RN8-51 decreased cancer cell growth with little apoptosis demonstrating itself as particularly promising for further anticancer therapeutic development. Kelley and colleagues synthesized novel, second-generation APE1 redox-targeted molecules such as APX2007, APX2009, APX2014, and APX2032 and determined whether they would be protective against neurotoxicity induced by cisplatin or oxaliplatin while not diminishing the platins' antitumor effect. Specifically, they used *ex vivo* model of sensory neurons in culture through which they demonstrated that APX2009 [(2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-N,N-diethylpentanamide] is an effective small molecule that is neuroprotective against cisplatin and oxaliplatin-induced toxicity. APX2009 also demonstrated a strong tumor cell-killing effect in tumor cells and the enhanced tumor cell killing was further substantiated in a more robust three-dimensional pancreatic tumor model [177]. Together, these data suggest that the second-generation compound APX2009 is effective in preventing or reversing platinum-induced CIPN while not affecting the anticancer activity of platins [177]. All three compounds (APX2007, APX2009, and APX2032) demonstrated similar inhibition of NF-κB binding.

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Finally, in 2010, Nyland and colleagues described a series of quinones including benzoquinone and naphthoquinone, analogues of the APX3330 with the ability to reduce tumor growth [189].

3.3. Targeting both the APE1 endonuclease and redox activities

A molecule that was shown to inhibit both APE1 endonuclease and redox function is Gossypol [190]. Gossypol is a natural polyphenolic aldehyde that exhibits various effects including antioxidant, anticancer, antiviral, antiparasitic, and antimicrobial activities. It can directly interact with APE1 and enhances the cell-killing effect of MMS and cisplatin. A recent clinical trial (ClinicalTrials.gov Identifier: NCT00540722) aimed to investigate the potential clinical benefit of combining Gossypol with docetaxel and cisplatin in patients with NSCLC who have high expression of APE1 [191]. The trial, designed as a prospective and randomized study, did not show a significant difference between the gossypol and the placebo group, although the gossypol-treated patients had better outcomes of increased progression-free survival (PFS) and overall survival (OS).

AT-101, a derivative of Gossypol and an oral inhibitor of the anti-apoptotic Bcl-2 and Bcl-xL proteins, has been shown to exhibit potent anticancer activity. However, its chemosensitizing effects are not fully understood. AT-101 enhanced the sensitivity of A549 cells to cisplatin *in vitro* and *in vivo* by inhibiting APE1-mediated IL-6/STAT3 signaling activation, suggesting its potential use in NSCLC chemotherapy [192]. It was also found to suppress gastric cancer cell migration and renewal and promote chemotherapeutic sensitivity in a gastric cancer model *in vivo* [193]. The molecular mechanism of its anticancer activity via inhibition of APE1 DNA repair and redox activities is still unclear.

4. Future perspectives from targeting the non-canonical roles of APE1 in miRNA processing

We recently proved that APE1 contributes to the expression of chemoresistance genes via functions in RNA metabolism involving miRs. We found that APE1: i) binds to structured RNAs, including pri-miRs [12,194]; ii) is involved in the processing of miRs implicated in cancer development (e.g. miR-221/222, miR-1246, miR-130b, miR-146a) [195]; iii) is involved in EVs-based sorting of specific miRNA through interaction with hnRNPA2/B1 (*in preparation*); iv) is a central hub connecting different subnetworks of cancer-associated proteins involved in RNA metabolism and miRs sorting (e.g. NPM1, hnRNPA2/B1, AUF1, FUS, SFPQ) [194,196,197]. We demonstrated that, during genotoxic stress, nuclear APE1 favors the processing and stability of miRs precursors through the association with DROSHA microprocessor complex, impacting, for example, the miR-221/222 axis and in turn, modulating the expression of the tumor suppressor PTEN [12]. Using NSCLC cancer cell lines, we recently defined a signature of 13 miRs (miR-1246, miR-4488, miR-24, miR-183, miR-660, miR-130b, miR-543, miR-200c, miR-376c, miR-218, miR-146a, miR-92b, and miR-33a) that strongly correlate with APE1 expression in human lung cancer and play a central role in cancer cell proliferation and survival [195]. Interestingly, bioinformatic analysis, demonstrated that these miRs possess a strong propensity to contain the RG4 structures in their immature or mature forms (*unpublished data*). Whether these APE1-regulated miRNAs are responsible for cancer cell response to genotoxic treatment, explaining the role of APE1 in chemoresistance through post-transcriptional mechanisms, is still unknown and should be addressed to understand the central role of APE1 in cancer progression and to define new antitumor strategies. It should be defined whether APE1 recognizes specific oncogenic miR alone or in combination with specific proteins through the detection of regulatory motifs present in the miR structure. On these lines, specific inhibitors of APE1-onco-miR interaction should be explored as potentially novel anticancer strategies for personalized medicine of cancer abs' on APE1-miR-specific signatures. Work is ongoing in our Lab along these new lines.

5. Secreted APE1 as a novel prognostic non-invasive biomarker of cancer development

We recently showed that APE1 can be secreted (sAPE1) by cancer cells through EVs, including exosomes, during genotoxic stress conditions and it is enzymatically active [8]. However, APE1

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presence in the extracellular milieu is still poorly characterized [186–188]. sAPE1 expression is actually considered as a novel biomarker for the prognosis of NSCLC as it was proved in a previous study performed on NSCLC patients, in which the levels of sAPE1 resulted significantly more elevated than in healthy controls and were associated with worse progression-free survival [196]. Recently, data obtained by our Lab, were confirmatory of these observations in a cohort of HCC patients [190], in which we found that sAPE1 levels correlated with poor prognosis and were able to discriminate between cancer patients and cirrhotic or healthy donors. The presence of this protein in sera of patients is not solely restricted to cancer diseases but also in inflammatory models, such as coronary artery disease and endotoxemia [191,192]. The biological function of sAPE1 is still completely unknown. An intriguing hypothesis could be that it might act as a paracrine molecule in triggering cell-to-cell communication, important for the local tissue microenvironment inflammatory response.

Also, evidence on the mechanisms responsible for APE1 secretion is lacking, even though the importance of the acetylation, occurring on specific lysine residues sited in the first 33 N-terminal portions of the protein (K27, K31, K32 and K35), has been highlighted in cells treated with the histone deacetylase inhibitor tricostatin A (TSA) [12] and our unpublished data. However, it seems reasonable that APE1 secretion might derive from EVs formation via endosomal sorting complex (ESCRT), due to the protein lacking of a classic secretory signal peptide [193]. This pathway is responsible for the biogenesis and maturation of multivesicular bodies (MVBs), composed of many intraluminal vesicles (ILVs), that are released in the extracellular milieu as exosomes. ILVs formation can occur through several mechanisms and information about the regulation of these processes and the possible differences between the promoted cargo selection is still missing [194].

It is conceivable that these vesicles might be highly shuttled between cells within tumor mass and deliver their content within target cells. This process may fulfill the cancer cells' requirement of a high amount of APE1 to counteract the DNA damage inferred by drugs in a paracrine manner, suggesting that APE1-secretion could represent a novel Damage-associated molecular pattern (DAMP) mechanism, that deserves further in-depth study to develop inhibitors that could specifically target alterations of APE1 secretion in different cancers.

6. Conclusive remarks and future perspectives

Albeit the high potency of many of the compounds aforementioned, additional work is necessary to deliver more specific inhibitors of APE1-altered functions in tumors, which could be useful for clinical trials. While progress has been certainly made in identifying potent APE1 inhibitors, further efforts are needed to specifically achieve selectivity and efficacy. This will require consideration of both the abasic site binding pocket and more distal features of the enzyme that might be important for DNA binding. X-ray crystallography and *in vivo* experiments would be crucial to expedite rational inhibitor design, validate APE1 as a target, and to explore possible side effects. Moreover, knowing the multiple APE1 cellular function and its relative detailed molecular mechanism could allow us to better target APE1 dysregulated in pathologies (Figure 2).

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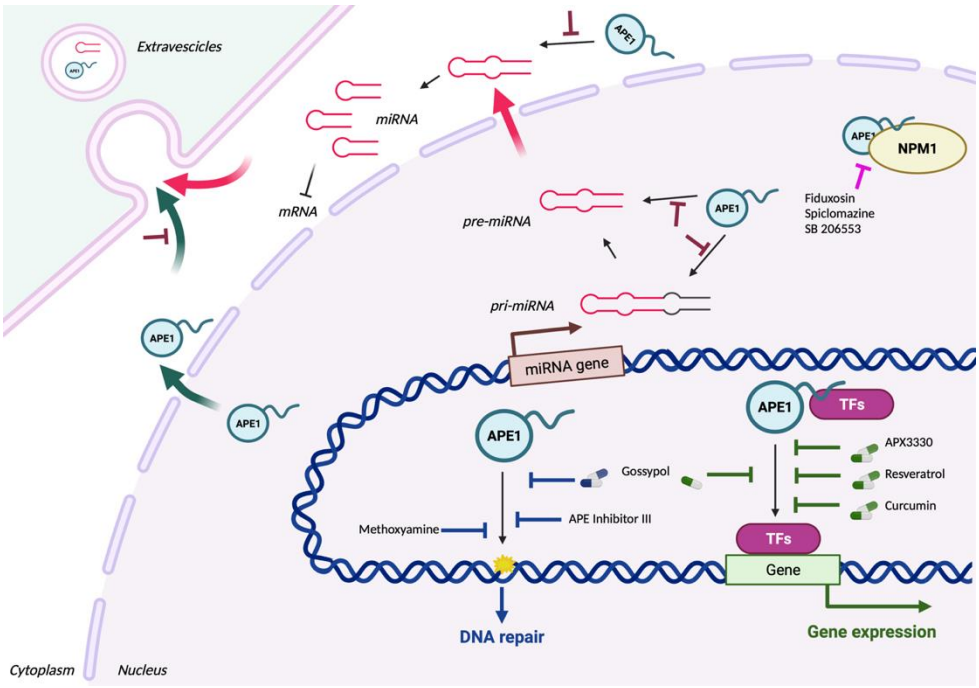

Figure 2. Illustration of the main functions of APE1 and relative inhibitors. The DNA repair mediated by the endonuclease activity of APE1 is inhibited by Methoxyamine, APE Inhibitor III, and Gossypol (blue blunt arrows). The gene expression promoted by the redox activity of APE1 on transcriptional factors (TFs) is inhibited by Gossypol, Curcumin, Resveratrol, and APX3330 (green blunt arrows). Especially through its N-terminal region, APE1 is involved in several PPI, including Nucleophosmin 1 (NPM1). This interaction is inhibited by Fiduxosin, SB 206553, and Spiclomazine (fuchsia blunt arrows). Recent findings have shown that APE1 is secreted by EVs in the microambient and is involved in RNA metabolism, including the regulation of miRNA processing. If these novel features of APE1 can be inhibited is still unknown (brown blunt arrows) and an interesting starting point for future explorations. For each inhibitor, pillows are drawn when the inhibitor has been enrolled in one or more clinical trials. Created with BioRender.com.

Targeting a protein-nucleic acid interaction is challenging and this has contributed to the limited success in developing APE1 inhibitors. New approaches are needed for the discovery of novel and selective APE1 inhibitors. In this context, Wilson DM III and colleagues proposed the application of Fragment- and structure-based drug discovery (FBDD/SBDD) methods in the quest for new clinical agents [198]. They applied the ABSOneStepTM platform identifying 25 high-quality crystal structures showing unique and diverse fragment hits bound at the endonuclease site, as well as at a previously unidentified secondary site, overall suggesting multiple novel strategies for inhibiting APE1. Indeed, in addition to direct inhibitors of APE1 nuclease activities, inhibitors against other functions of APE1 may also be clinically valuable. Considering the complex role of APE1, exploring allosteric modes of inhibition, such as disrupting vital interactions between APE1 and other cellular protein partners might be an alternative option [116,199]. For example, we demonstrated that the molecular association with nucleophosmin (NPM1) modulates the endonuclease activity of APE1 [116]. HTS for the disruption of this interaction led to the discovery of three compounds (fiduxosin, spiclomazine and SB 206553); of these, fiduxosin and spiclomazine displayed anti-proliferative activity and sensitized cells to bleomycin. A synergistic effect with platinum drugs was also observed by using these inhibitors in triple negative breast cancer cell model, demonstrating how APE1 could represent a useful therapeutic biomarker also in this type of tumor [47,200]. Similarly, disruption of other APE1 protein interactions or functions can be taken into consideration.

RNA G-quadruplexes (RG4s) are disease-associated non-canonical structures, composed of stacks of guanine tetrads (called G-quartets) kept together by Hoogsteen hydrogen bonds. RG4s are increasingly recognized as fundamental post-transcriptional regulators of gene expression [201].

Interestingly, these elements are widespread in the transcriptome and are particularly enriched in miRs [202]. The folding of these structures can be controlled by their RBP interactors (i.e. hnRNPA2B1, FUS, etc.), cations (i.e. K^+), and small molecule ligands [203], making RG4s highly dynamic. Very recent data underline a regulatory function played by RG4 in miR-maturation through Dicer-inhibition [204] and a potential role in physiological and pathological liquid-liquid phase separation [205,206]. The presence of the rG4 structure in pre-miR exists in equilibrium with the canonical stem-loop structures and this equilibrium regulates the maturation of some miR, such as miR 92b [207]. However, mechanistic information on RG4 function in miR sorting is missing as well as information on the functional role of oxidized guanine (8-oxo) or abasic (AP) sites in the RG4 forming structures, in the stability and biological properties of the miRs in which these structures are present. Understanding whether APE1 function on miR-processing and -decay could be driven by RG4-mediated folding will open mechanistic views as well as translation applications in cancer biology. We are working along these lines.

A fascinating new field of research relies on the unexpected finding that APE1 can be secreted in the extracellular milieu through EVs. Understanding the intracellular routes responsible for the secretion processes in cancer cells and the role of sAPE1 as a potential paracrine molecule will open new perspectives on precision Medicine.

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