The Cyclin-Dependent Kinase Inhibitor p21^{CDKN1A} as a Target of Anti-Cancer Drugs

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Abstract: p21^{CDKN1A} (WAF1/CIP1/SDI1), the cyclin-dependent kinase (CDK) inhibitor belonging to the Cip/Kip family, was first described as a potent inhibitor of cell proliferation and DNA replication, both in physiological conditions and after DNA damage. More recently, p21 has been recognized to play additional and fundamental roles in other important pathways, including regulation of transcription, apoptosis and DNA repair. Knock-out mouse studies combined with biochemical and functional analysis of cells in culture have indicated a tumor suppressor activity for p21. However, these lines of evidence have been complicated by other findings indicating that p21 can exhibit oncogenic properties. In fact, the evidence that p21 expression may lead to proliferation arrest, is counterbalanced by the rescue of tumor cells from drug-induced apoptosis, and by promoting a metastatic potential. For these reasons, p21 is considered a protein with a dual behavior, with potential benefits, as well as dangerous effects of its expression in malignant cells. Thus, the effectiveness of targeting p21 expression for antitumor therapy needs to be carefully evaluated accordingly. This review summarizes the functions and regulations of p21, and focuses on its involvement in human diseases (particularly cancer), and on the pharmacological approaches to target p21 expression (either positively or negatively) for anticancer therapy. Based on these approaches, the search for new molecules that are able to promote the tumor-suppressor activity, and/or to interfere with the oncogenic properties of p21, could be promising.

Keywords: Anti-cancer target, CDK inhibitor, cell cycle regulator, p21^{CDKN1A}.

INTRODUCTION

p21^{CDKN1A} is a conserved protein, belonging to the CDK inhibitor Cip/Kip family, including also p27 and p57, which is implicated in the cell cycle regulation of several organisms. Homologs are found in *Xenopus* (Xic1), in *Drosophila* (Dacapo), as well as in *C. Elegans* (CKI-1). In mammals, p21^{CDKN1A} is known as CDK-interacting protein 1 (CIP1), wild type p53-activated fragment (WAF1), senescent cell-derived inhibitor 1 (SDI1), and melanoma differentiation-associated protein 6 (MDA-6). It was the first CDK inhibitor identified, almost simultaneously by different groups, with diverse approaches [1-3]: it was discovered as a CDK2-associated protein in a yeast two-hybrid system, and it was biochemically isolated as a cyclin-CDK2 binding protein [1]. In addition, p21 was discovered as a growth inhibitor in human glioblastoma, and lung adenocarcinoma cell lines with activated p53 [2], or in senescent human diploid fibroblasts [3]. The early studies indicated that p21 has a tumor suppressor role by inhibiting cell proliferation in response to a variety of endogenous, as well as exogenous stimuli, through p53-dependent or independent mechanisms, to promote or inhibit multiple biological functions (Fig. **1**). However, more recent studies have also shown that p21 may favor cell survival and proliferation, with a consequent oncogenic potential [4]. This contrasting behavior has been shown to depend on a surprising complexity of protein-protein interactions and networks in which this relatively small protein is involved.

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The idea that increasing p21 protein levels could lead to proliferation arrest, has been initially considered to exploit this p21 function in neoplastic cells. However, the opposite strategy, *i.e*. targeting p21 expression in order to abolish its oncogenic activity, is also being pursued. Thus, different types of approaches, *i.e.* those trying to induce p21 expression *vs* those aiming at its down-regulation, are under investigation.

p21 BIOLOGY

p21 Functions

p21 participates in important cellular pathways, playing diverse functions in the regulation of fundamental biological processes, such as: i) cell cycle, ii) DNA replication/repair, iii) gene transcription, iv) apoptosis, and v) cell motility (Fig. **2**), which are briefly summarized hereafter.

Cell Cycle Regulation

The main role of p21 in cell cycle regulation lies in its ability to inhibit the activity of CDK2 in complex with cyclin E (or with cyclin A), thereby contributing to G_1 phase arrest [5]. The primary activity was first demonstrated in the cell response to DNA damage, through induced expression by p53 [1, 6, 7], thereby preventing initiation of DNA replication at the G1/S checkpoint [7]. Accordingly, mouse embryonic fibroblasts (MEFs) obtained from p21-null mice failed to arrest in G1 phase, in response to DNA damage [8, 9].

It has been shown that p21 is also essential to sustain the G2 phase checkpoint after DNA damage in human cells, as well as in preventing the G2-arrested cells from undergoing additional S-phase [10-12].

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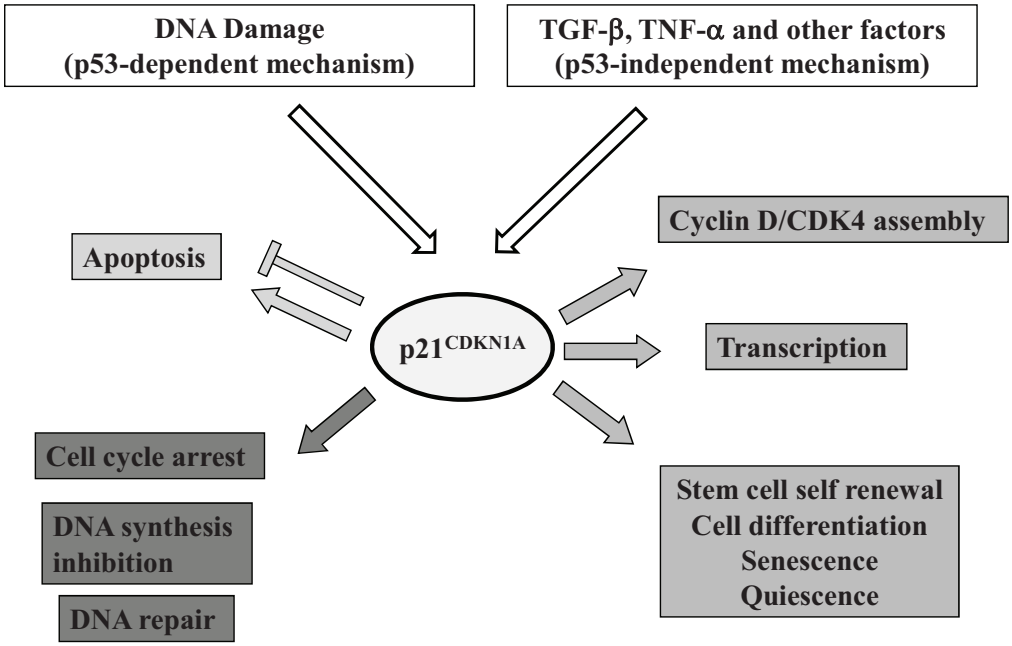

Fig. (1). Biological functions in which p21^{CDKN1A} is involved. Under physiological condition, p21 is expressed at low levels, promoting cell cycle progression through the assembly of cyclin D/CDK4 complex. Upon exposure to a variety of stimuli (DNA damage, oxidative stress, cytokines and other factors), p21 expression is increased through p53-dependent and -independent mechanisms. Increased p21 levels trigger various responses such as cell cycle arrest and DNA synthesis inhibition, thus allowing DNA repair, apoptosis, senescence, differentiation, and quiescence.

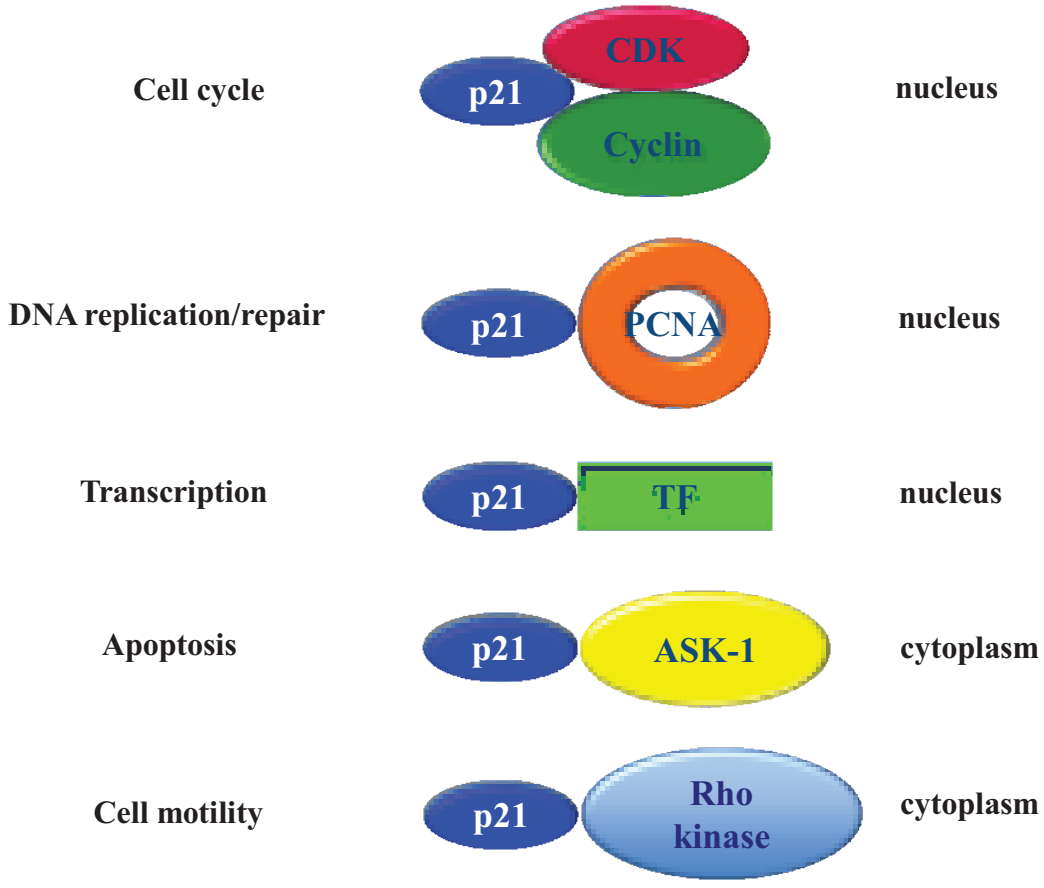

Fig. (2). Schematic representation of the main protein interactions established by p21^{CDKN1A} and mediating its functions. Main pathways and processes requiring the participation of p21 with proteins described, are reported on the left, while the cellular compartment in which these regulatory interactions occur, is shown on the right.

p21 is also involved in the basal proliferation control of specific cell types. In particular, the stem cell self-renewal of keratinocytes [13] of the hematopoietic system [14], and of the mouse forebrain and hyppocampus [15, 16], have been shown to depend on p21 protein. In fact, studies in *CDKN1A* knock-out mice showed that p21 restricts the self-renewal potential of stem cell population, and promotes their irreversible commitment to differentiation [13]. In the absence of p21, an increase in stem cell proliferation with a consequent exhaustion of the population was observed in different cell types [13-17]. Interestingly, p21 is also able to maintain the self-renewal potential of leukemic stem cells, and to protect them from DNA damage accumulation, thereby demonstrating an oncogenic activity of the protein [18].

Cell quiescence and senescence are other phenomena in which p21 plays a fundamental role by keeping cells arrested in G0, or G0-like state, in order to prevent untimely DNA replication [4, 19]. In fact, loss of p21 has been shown to facilitate cell cycle entry from a quiescence state, at the expense of replication stress [20]. Interestingly, lack of p21 expression has been found to link cell cycle control with appendage regeneration in mice, since $p21^{-/-}$ animals showed a phenotype similar to that of regenerating mouse strains [21].

p21 also plays a complex role in cell differentiation. In fact, its expression is induced in differentiating cells of the skin and of the intestinal epithelium, as well as in cultured epidermal cells, while down-regulation has been observed at late stages of differentiation [22, 23]. However, p21 appears to play a positive role in promoting differentiation of human promyelocytic leukemia cells [24], mouse skeletal muscle and cartilage cells [25, 26], and oligodendrocytes [27]. The emerging consensus is that p21 plays either positive or negative roles in differentiation, independently of cell cycle control, but depending on cell type and specific stage of differentiation. This regulatory function may involve specific interactions of p21, *i.e.* with caspase-3 [28], or with calmodulin [29], as critical regulators of keratinocyte differentiation [22].

In contrast with the CDK inhibitory function, a cell growth promoting effect has also been demonstrated [30]. In fact, p21 may serve as an assembly factor for cyclin D/CDK4 complex, thereby promoting its nuclear translocation, kinase activation, and cell proliferation [30]. This function has been suggested to potentially contribute to the oncogenic activity of p21 [31, 32].

DNA Replication and Repair

The activity of p21 in the G1/S checkpoint may also occur through direct interaction with the Proliferating Cell Nuclear Antigen (PCNA), a ring-shaped protein which provides a molecular platform for coordinating the activity of many factors involved in DNA transactions [33, 34]. Through high affinity binding to PCNA, p21 inhibits *in vitro* DNA synthesis by dissociating the interaction of replicative protein partners (*e.g.* DNA polymerase δ) with PCNA [35]. At the cell level, such a mechanism has been reported only in over-expression experiments [36], being difficult to observe under normal conditions, probably because p21 is degraded in S phase [37].

Another inhibitory function for p21, although controversial, was envisaged in DNA repair and, for this reason, degradation of p21 was suggested to be necessary for DNA repair [38]. However, different approaches have led to contrasting results, particularly regarding the inhibition of nucleotide excision repair (NER). Notably, several lines of evidence has shown that p21 does not interfere with NER, and that deletion of *CDKN1A* gene reduces DNA repair efficiency in normal human fibroblasts [reviewed in 39]. In fact, p21 has been shown to regulate the acetyltransferase activity of p300 in NER, by dissociating the inhibitory interaction of PCNA [40]. The involvement of p21 in other pathways of DNA repair has been shown in single- and double-strand break repair, and in base excision repair (BER), where p21 regulates the interaction of poly(ADP-ribose) polymerase 1 (PARP1) with other BER factors [41]. In addition, p21 has been shown to contribute to translesion DNA synthesis (TLS) by regulating PCNA ubiquitination, thereby limiting mutagenic load [42, 43]. By integrating the multiple roles of p21 in the DNA damage response, a model has been proposed in which p21 protein will favor DNA repair in the presence of low levels of genotoxic lesions. In contrast, p21 will be degraded in the presence of extensive DNA damage, to favor apoptotic cell death [39].

Transcriptional Regulation

Besides its classical role as CDK inhibitor, p21 functions as a transcriptional co-factor that may regulate transcription, either positively or negatively. This activity of p21 may occur through three different mechanisms: i) by inhibition of cyclin/CDK complexes, ii) by direct binding to several transcription factors, such as NF-kB, Myc, E2F, STAT3, and estrogen receptors, iii) by regulating the activity of transcriptional co-factors, such as p300/CBP [44-46]. In case of the first mechanism, inhibition of CDK is relevant to the phosphorylation of RB and consequent regulation of E2F-mediated transcription. In the second mechanism, p21 acts as a co-factor that physically interacts with, and represses the activity of transcription factors. Thus, p21 may simultaneously target growth-promoting genes, such as *c-myc* and *CDC25A,* in addition to CDK activity, to induce cell cycle arrest [47]. However, in the case of estrogen receptors (ER), the binding of p21 to ERα results in an increase in transcriptional activity, thanks to concomitant recruitment of CBP to a target gene [48]. The third mechanism occurs by modulation of a repression domain in p300, which occurs independently of the CDK inhibitor effect on p300 phosphorylation [49].

Apoptosis

p21 is a major inhibitor of p53-dependent as well as p53-independent apoptosis. In fact, reduction in p21 expression, together with the presence of a cleaved fragment of the protein, was shown to lead to apoptosis in DNA-damaged human cancer cells. The cleavage and inactivation of p21 are mediated by caspase-3 in human normal and cancer cell lines [50, 51]. A number of different signals (such as TGF-β, TNF-α, IFN-γ, and IL-6) inducing p53-independent

expression of p21 in several human cell lines, induce not only cell cycle inhibition, but also suppression of apoptosis [51, 52].

Two mechanisms of action are responsible for this phenomenon: *i*) the interaction with pro-apoptotic regulatory proteins, such as pro-caspase-3, caspase-8 or apoptosis signal-regulating kinase-1 (ASK-1), with their consequent inhibition [53-55]. *ii*) The inhibition of apoptotic events, such as chromatin condensation, cell shrinkage and loss of adhesion, by targeting caspase-dependent activation of CDKs [56].

In contrast with the anti-apoptotic role, p21 appears to possess pro-apoptotic functions under certain conditions, and in specific systems [reviewed in 57]. In fact, p21 overexpression in thymocytes induced hypersensitivity to p53-dependent cell death in response to X-rays and UV radiation [58]. Overexpression of p21 was shown to enhance the apoptotic response to cisplatin in glioma and ovarian carcinoma cell lines [59, 60]. Similarly, p21 promoted C_6-ceramide-induced apoptosis in a p53-deficient human hepatoma cell line [61]. In addition, introduction of a p21-expressing adenovirus into a panel of p53-deficient cervical cancer cell lines resulted in both growth inhibition and apoptosis [62]. Other studies reported the pro-apoptotic role of p21 after targeted overexpression of the protein [63-65], or by showing a decrease in apoptosis after p21 gene disruption [51]. However, the mechanism(s) by which p21 may promote apoptosis are still to be clarified.

Interestingly, p21 may also play an important role in regulating another type of cell death, *i.e.* autophagy, a process in which cell organelles are enclosed and destroyed in vesicles [66]. This mechanism appears to be regulated by p21 by maintaining autophagic proteins in an inactive state [67].

Cell Motility

One of the most recently described functions of p21 is the regulation of actin-based cell motility. Through the inhibition of Rho kinase, cytoplasmic p21 has been shown to influence cell motility and neuronal neurite outgrowth by interfering with substrate adhesion [68]. Degradation of cytoplasmic p21 favors a nonmotile cell behavior. In tumor cells, high levels of p21 localized in the cytoplasm will favor Rho inhibition with consequent enhanced cell movement [69]. This effect has been shown to contribute to tumor metastasis and invasion, thus suggesting another mechanism by which p21 plays an oncogenic role [31, 32].

p21 Localization

The intracellular localization of p21 has been shown to be fundamental for determining the target of p21, and consequently to direct its activity. In fact, early studies indicated that lack of p21 expression, or cytoplasmic localization of the protein, promoted anchorage-independent growth [70, 71], and drug resistance [31, 32]. Human p21 protein is located predominantly in the nucleus, however, it is also present in the nucleolus, and also in the cytoplasm. In the nucleus, in addition to inhibiting CDK2 and PCNA, p21 may also bind to transcriptional regulators [45]. The nucleolar p21 was found to co-localize with cyclin E [72], and it was shown to accumulate after DNA damage, as a consequence of inhibition of nuclear export [73]. As stated above, p21 in the cytoplasm may interact with, and inhibit ASK-1 and pro-caspase 3 to prevent apoptotic cell death [53-55, 74, 75], and also with Rho kinase, thereby contributing to cell motility [68]. Interestingly, increasing number of evidence indicates that the cytoplasmic localization of p21 is linked to drug resistance [31, 32], thus suggesting that in this compartment the tumor-promoting activities are favored.

p21 localization is regulated mainly by post-translation modifications. Thus, nuclear translocation appears to be counteracted by different kinases phosphorylating Thr^{145} and Ser^{146} residues located near the nuclear localization signal (NLS) region of p21 [76-78]. These modifications are responsible for cytoplasmic localization of p21, as well as for loss of interaction with PCNA [79]. An important role is played by AKT1/PKB in p21 phosphorylation, thereby mediating its localization and stability [76, 77]. In fact, another important modification of p21 (*i.e.* ubiquitination) regulating its degradation, has been shown to occur predominantly in the nucleus, because p21 mutant in the NLS region exhibited enhanced stability [80]. Regulation of nuclear translocation of p21 was found to depend on interaction with calmodulin [29].

p21 Regulation

Transcriptional control of p21 expression, either by p53-dependent or independent mechanisms, has been extensively reviewed [32, 39, 44, 45]. Additionally, p21 expression can be regulated both at the post-transcriptional and post-translational level. In fact, the regulation of p21 mRNA stability is mediated by noncoding microRNA, and by RNA binding proteins [81-83]. Protein stability is strictly regulated mainly by phosphorylation [reviewed in 84], and protein degradation is performed through ubiquitin-dependent and independent mechanisms [39, 85]. A number of residues in p21 have been identified, and found to be targeted by different protein kinases [84]. Phosphorylation can have important consequences for p21 function, such as modulation of binding partner and changes in subcellular localization (as discussed earlier), with concomitant modification of p21 stability. Phosphorylation at Ser^{130} by Cyclin E/CDK2, for example, promotes binding to SKP2, allowing p21 ubiquitylation and subsequent proteolysis [86], in contrast, stress activated kinases p38a and JNK1 were shown to stabilize p21 by phosphorylation at Ser^{130} [87]. Phosphorylation at Thr^{145} in the PCNA-binding motif, disrupts the binding with PCNA, and induces cytoplasmic accumulation [79, 80]. The transcription factors C/EBPα and C/EBPβ were found to interact with p21 protein and protect it from proteolytic degradation [88].

Ubiquitination represents the other important post-translational modification of p21, that triggers the mechanism driving protein degradation *via* proteasome complex [89], both under normal growth, and in response to DNA damage. Three E3 ubiquitin ligase complexes, SCF^{SKP2}, $CRL4^{CTD2}$, and APC/C^{CDC20} promote p21 proteolysis through the proteasome at specific stages during cell cycle [reviewed in 39]. Ubiquitin-independent

mechanisms of p21 proteolysis have been also reported, which are the direct association with the C8α subunit of 20S proteasome [90], or with MDM2 [91, 92]. Finally, N-terminal acetylation of p21 has been also shown to influence its degradation [93].

p21 AND DISEASE

Current knowledge on the involvement of p21 in disease, and particularly in cancer, has been initially obtained from experimental models, especially knockout mice (Table **1**), which were developed to investigate p21 protein function [8, 94]. These mice developed normally and did not show tumor formation until 7-months age [8]. However, subsequent studies reported that CDKN1A-null mice showed an increased tumor formation at a median age of 16 months, with the most common tumor types being sarcomas and B-cell lymphomas [94]. In addition, accelerated tumor formation and increased tumor metastasis, respectively induced by urethane, or by gamma radiation, were found in $p21^{-/-}$ mice [95, 96]. Accelerated tumorigenesis and promotion of lung metastasis were also found in correlation with cytoplasmic p21 in the mammary epitelium of MMTV/*neu* expressing mice [97]. Tumor suppression functions of p21 were also shown by studies in the skin and in the colon of p21-deficient mice [98, 99]. Furthermore, p21-null mice were also found to exhibit spontaneous tumor formation in other knock-out genetic background, such as $Muc2^{-/-}$ (lacking mucin 2), and $Apc^{1638+/-}$ (mutant allele of the adenomatosis polyposis gene) mice [100, 101].

In addition to enhanced tumor formation, further investigations showed that loss of p21 caused exhaustion of blood stem cells [14], and induced development of systemic lupus erythematosus in female animals [102]. Interestingly, *CDKN1A*-null mice (crossed with knock-in PML-RAR mice) showed an oncogenic role of p21 in maintaining self-renewal of leukemic stem cells [18]. Transgenic mouse models have been also developed. Mice expressing human *CDKN1A* transgene, restricted only to T cells, were generated for studying radiation-induced apoptosis [58]. Transgenic mice overexpressing *CDKN1A* were developed for studying control of the proliferation/differentiation balance of erythroid and megakaryocytic progenitors [103]. A targeted knock-in transgenic mouse developed by the integration of genes encoding for Hepatitis B virus surface antigen and X protein into the *CDKN1A* locus, showed hepatocarcino-genesis [104].

Cellular model systems in which *CDKN1A* gene had been deleted by homologous recombination, have been developed from primary cultures of human fibroblasts [105], and from the human colon cancer cell line HCT116 [6, 10, 12]. Both cell types have been found defective in G1 and G2 checkpoints. In addition, $CDKN1A^{-/-}$ human fibroblasts have shown senescence bypass [105], defects in DNA repair [106], as well as impaired ability to enter quiescence [20].

Existing data indicate that p21 protein plays a significant role in human tumor suppression. In fact, homozygous deletions in *CDKN1A* have been detected in human melanoma cell lines and, though less frequently, in melanoma cancer tissue samples [107]. A significant correlation between p21 expression and melanoma differentiation has also been reported [107], suggesting that the loss of protein function may be associated with melanoma tumorigenesis, progression and metastatic phenotype [108].

Evidence has also been provided on the association between polymorphisms in the *CDKN1A* gene and human esophageal cancer [109], prostate carcinoma [110] or breast cancer [111], although a higher number of cases need to be tested in order to confirm these results. However, it must be noted that significant controversy has been reported in the clinical association between p21 expression and cancer, as investigated in several tumor types [reviewed in 31], which may be explained by the complexity of tumor phenotypes, including p53 status, or p21 cytoplasmic localization [112].

Since *CDKN1A* is an important gene involved in multiple levels of the cell response to DNA damage [39], it appears that all existing models are undoubtedly useful for studying the involvement of p21 protein in proliferation-associated diseases (*i.e.* tumors), and they may also be useful for studying other diseases. An example is provided by the p21 function linked to the immune system, particularly by

Table 1. Correlation between p21 gene alterations and diseases.

Disease	p21 gene alterations	Refs
Sarcoma	Homozygous deletion in mice	[94]
B-cell lymphoma	Homozygous deletion in mice	[94]
Melanoma	Homozygous deletion in melanoma cell lines, reduced expression in primary and metastatic human melanoma cells	[107,108]
Esophageal neoplasms	Polymorphisms in tumor tissue specimens	[109]
Prostate neoplasms	Polymorphisms in tumor tissue specimens	[110]
Breast ductal neoplasm	Mutation Arg94 → Trp in tumor tissue specimens	[111]
Mouth neoplasms	Polymorphisms in tumor tissue specimens	[109]
Systemic lupus erythematosus	Deletion in mice	[102]
Leukemia	Ectopic expression in leukemia cell line	[115]
Rheumatoid arthritis	Reduced expression in rheumatoid arthritis synovial lining	[114]

controlling excessive T-cell proliferation. Absence of this activity in p21-deficient mice lead to an abnormal accumulation of $CD4^+$ memory cells, and to loss of tolerance towards nuclear antigens, lymphadenopathy and glomerulonephritis. These findings indicated a specialized role for p21 in preventing lupus-like disease in female mice [102]. In addition, a protecting effect of p21 in focal segmental glomerulosclerosis was demonstrated after podocyte injury [113]. A novel role of p21 has been recently demonstrated in rheumatoid arthritis [114]. Loss of p21 expression occurs in fibroblast-like synoviocytes (FLS) isolated from patients with rheumatoid arthritis, and FLS from *CDKN1A*-deficient mice grow more rapidly and display a marked increase in migration compared to wild type cells. These data indicate that loss of p21 expression may contribute to excessive invasion and subsequent joint destruction. Thus, based on these findings, it appears that p21 could represent a therapeutic target not only for tumors, but also for autoimmune diseases. Further work is, however, necessary in order to fully elucidate disease-causing mechanisms involving p21.

p21 AS A CANCER DRUG TARGET

Approaches to Increase p21 Expression

Based on its activity as cell cycle inhibitor, the idea that increasing p21 protein levels could lead to proliferation arrest in neoplastic cells, was considered in early studies [reviewed in 31, 32]. The possibility of using gene therapy for leukemia was investigated in a human leukemia cell line by inducing p21 expression through a retroviral vector. The results showed that p21 inhibited the proliferation by arresting cells in the G0/G1 phase [115].

Exploiting the increased cellular levels of the protein achieved by drug treatment, two different experimental strategies, are currently under investigation (Table **2**). The first is based on the induction of p21 expression by using histone deacetylase (HDAC) inhibitors [116, 117] and the second one exploits the accumulation (reduced turnover) of p21 induced by proteasome inhibitors [118, 119].

HDAC inhibitors have been shown to de-repress a subclass of genes, including p21, involved in cancer cell growth [120-122]. Compounds in this class typically induce expression of p21, which subsequently results in growth inhibition and differentiation of cancer cells [121, 123]. From these compounds, drugs have been developed such as Vorinostat (suberoylanilide hydroxamic acid, SAHA), Romidepsin (Depsipeptide, FK-228), Valproic acid, Belinostat (PXD101), Panobinostat (LAQ824/LBH589), and Givinostat (ITF2357) [117, 124, 125]. These drugs have been shown to induce p21 expression in a variety of tumor cells [126-135], and have demonstrated therapeutic benefit in cutaneous T-cell lymphoma as well as in other malignancies, including solid tumors [117, 121]. Novel HDAC inhibitors have also demonstrated therapeutic potential in monotherapy, or in combination with other anticancer drugs, both in hematological and solid malignancies [136-138].

Pharmacologic inhibitors of the proteasome have been shown to possess *in vitro* and *in vivo* antitumor activity [118, 119]. The first such agent to undergo clinical testing, i.e. Bortezomib (Velcade), demonstrated significant efficacy against cell lines derived from non-Hodgkin's lymphoma, and also in human mesothelioma and breast cancer cells [139, 140]. MG-132, MG-115, Lactacystin, and Epoxomicin are representative examples of other proteasome inhibitors investigated so far [118]. All of them act by stabilizing the levels of proteins involved in checkpoints (*e.g.* p53 and p21), and in apoptotic pathways (*e.g.* bax). Therefore, these drugs are not direct specific effectors of p21. Bortezomib was indicated to be active in the treatment of hematologic and also solid malignancies [141, 142]. MG-132 has been shown to have activity against gastric adenocarcinoma cells and follicular thyroid cancer cells [143, 144].

Full potential of both classes of inhibitors is still under evaluation, and their usefulness as anti-cancer agents needs to be determined by the careful analysis of gene expression changes. Such studies, in monotherapy or combined with conventional chemotherapy, might lead to synergistically improved response and toxicity for an enhanced therapeutic benefit to the patient [142].

A number of clinical trials (www.clinicaltrials.gov) proposing the use of drugs inducing the expression of p21, are actively running. Drugs used in these trials include Vorinostat (alone or in combination) for non-small lung cancer, glioblastoma multiforme, and advanced solid tumors. Belinostat is used for metastatic thymoma and thymic carcinoma. Bortezomib is used in trials on urothelial cancer and solid tumors. In all these trials, p21 expression is taken as a marker of the drug effect.

Based on the different rationale of exploiting its pro-apoptotic activity, p21 induction or stabilization by proteasome or HDAC inhibitors, respectively, is currently used or investigated for the treatment of hematological and solid malignancies. These include multiple myeloma, leukemia and lymphoma, as well as prostate, pancreatic, ovarian, breast e colorectal tumors. In fact, both proteasome and HDAC inhibitors have been shown to upregulate p21 and to induce apoptosis in multiple myeloma cells [116, 140].

It is worth noting that different approaches to increase p21 levels with small molecules acting through p53-MDM2 dependent (*e.g.* nutlin) [145], as well as independent mechanisms [146, 147], are also being investigated.

Approaches to Decrease p21 Expression

Given that p21 appears to have also an oncogenic potential [148] attempt to down-regulate p21 expression has been taken into consideration [149]. A recent report investigating the role of p21 in DNA repair and in the anti-apoptotic pathways, has shown the possibility that increase in p21 protein induced by chemotherapeutic drugs, would allow human cancer cells to escape death and to repair damaged DNA [149]. Attenuation of p21 levels may subvert this process, making such drugs more effective. Thus, p21 could be a therapeutic target in breast and other cancers characterized by chemotherapy resistance [149]. In fact, p21 deletion has been shown to sensitize tumor cells to antiproliferative drugs [12, 150]. In this regard, several

Table 2. Therapeutic agents targeting p21^{CDKN1A} in clinical and research studies.

Agents	Tumor cells	CDKN1A expression	References
HDACIs			
Vorinostat (SAHA)	CTCL, NHL, GBM	Increased expression	[125]
Romidepsin or Depsipeptide (FK288)	CTCL, AML, LC, NB, PC	Increased expression	[126, 127, 128, 129]
Belinostat (PXD101)	BLC	Increased expression	[130]
Valproic Acid	GC, PC, HNC	Increased expression	[131, 132,133]
Panobinostat (LBH589)	CTCL, HL	Increased expression	[134]
Givinostat (ITF2357)	MM, AML, HL	Increased expression	[135]
Proteasome inhibitors			
Velcade (Bortezomib)	HL60, MM, MC, NHL	Increased expression	[139,140,142]
MG-132	BRC, FTC, TMK1,	Increased expression	[140,143, 144]
Antisense oligos	HL60, RC, BRC	Decreased expression	[151, 152, 153]
RNA interference	MC, TC	Decreased expression	[154, 155]

Abbreviations - AML: Acute myelogenous leukemia, BLC: bladder cancer, BRC: breast cancer, CC: colon cancer, CTCL: cutaneous T-cell lymphoma, FTC: follicular thyroid cancer, GC: gastric carcinoma, GBM: glioblastoma multiforme, HL: Hodgkin's lymphoma, HL60: human promyelocytic leukemia cells, HNC: head and neck cancers, LC: lung carcinoma, MDS: myelodysplastic syndrome, MM: multiple myeloma, MC: mesothelioma-derived cells, NB: neuroblastoma, NHL: non-Hodgkin's lymphoma, PC: prostate cancer, RC: renal carcinoma, TC: testicular cancer, TMK1: adenocarcinoma gastric cells.
Vorinostat and Bortezomib approved for the treatment of CTCL by US Food and Drug Administration (FDA).

studies performed on different human cell model systems, including myeloid leukemia [151], renal carcinoma [152], and breast cancer cells [153], have used antisense oligonucleotides to suppress or attenuate p21 expression. Although the use of these agents has potential clinical applicability, it still awaits further investigations [57, 149].

Experimental approaches directed towards silencing p21 expression by RNA interference, have also been investigated on human cells of pleural mesothelioma [154]. Recently, it has been observed that high cytoplasmic p21 expression is related to cisplatin resistance in testicular embryonal carcinoma [155]. This resistance appears to be modulated by Oct4 and miR-106b seed family members, thus suggesting new strategies using synthetic anti-miRNA for the treatment of chemoresistant testicular, and other types of cancer.

Approaches Targeting p21 Cytoplasmic Localization

Since cytoplasmic localization of p21 has been shown to confer a tumor promoting phenotype, mainly due to drug resistance associated with inhibition of apoptosis [31, 32], it has been suggested that interfering with this behavior would lead to cell sensitization to anticancer drugs. Being p21 cytoplasmic localization influenced by phosphorylation, targeting p21 kinases (*e.g.* with LY294002 inhibitor of AKT1) would result in nuclear protein relocation, and consequent reversal of apoptosis-mediated drug resistance [155]. Therefore, approaches based on molecules able to interfere with p21 cytoplasmic localization, would in theory reach the double goal of arresting cell cycle and restoring apoptotic cell death.

CONCLUSIONS AND PERSPECTIVES

From current literature no drugs directly targeting p21 protein (*e.g.* small molecules inhibiting its function through binding and/or sequestration) appear to be available, most probably because p21 has been considered for a long time only a cell cycle inhibitor. However, high throughput screening has been recently applied to search small molecules to be used as attenuators of p21 as chemotherapy sensitizers [156]. The multiple functions of p21 in the DNA damage response likely contribute to its activity of tumor suppressor [36]. In particular, the ability to induce cell cycle arrest and to participate in DNA repair processes, can protect cells from stress-induced apoptosis. However, due to its anti-apoptotic activity, p21 is also believed to act as an oncogene. Thus, the "antagonist duality" of p21 is explained by the evidence that the anti-cancer effects (*e.g.* anti-proliferative activity) are counteracted by the pro-cancer effects (inhibition of apoptosis, ability to repair DNA in tumor cells, and promotion of a metastatic potential). The prevalence of each effect will likely depend on p21 cellular localization, the cell type and the biological environment [57]. Therefore, the tumor behavior with respect to p21 expression level, should be known in advance for proper chemotherapy choice, that should then be based either on induction or silencing of protein expression. Because loss of this CDK inhibitor usually increases sensitivity of tumor cells resistant to apoptosis induced by chemotherapeutic agents, small molecules that reduce p21 expression or interfere with its cytoplasmic localization, might improve the efficacy of anticancer drugs [156], by abrogating both the inhibitory effect on apoptosis, and the tumor-promoting influence on cell motility.

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ABBREVIATIONS

ASK-1	=	Apoptosis signal-regulating kinase 1
BER	=	Base excision repair
CDK	=	Cyclin-Dependent Kinase
CIP1	=	CDK Interacting Protein 1
ER	=	Estrogen receptor
HDAC	=	Histone Deacetylase
HDACI	=	Histone Deacetylase Inhibitor
IFN	=	Interferon
MDA-6	=	Melanoma differentiation-associated protein 6
MMTV	=	Mouse mammary tumor virus
NER	=	Nucleotide excision repair
NLS	=	Nuclear localization signal
PARP-1	=	Poly(ADP-ribose) polymerase 1
PCNA	=	Proliferating cell nuclear antigen
SDI1	=	Senescent cell-derived inhibitor 1
TGF	=	Transforming growth factor
TF	=	Transcription factor
TLS	=	Translesion DNA synthesis
TNF	=	Tumor necrosis factor
WAF1	=	Wild type p53-activated fragment 1

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