Content

When the guardian sleeps: Reactivation of the p53 pathway in cancer

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Abstract

The p53 tumor suppressor is inactivated in most cancers, thus suggesting that loss of p53 is a prerequisite for tumor growth. Therefore, its reintroduction through different means bears great clinical potential. After a brief introduction to current knowledge of p53 and its regulation by the ubiquitin-ligases MDM2/MDMX and post-translational modifications, we will discuss small molecules that are able to reactivate specific, frequently observed mutant forms of p53 and their applicability for clinical purposes. Many malignancies display amplification of *MDM* genes encoding negative regulators of p53 and therefore much effort to date has concentrated on the development of molecules that inhibit MDM2, the most advanced of which are being tested in clinical trials for sarcoma, glioblastoma, bladder cancer and lung adenocarcinoma. These will be discussed as will recent findings of MDMX inhibitors: these are of special importance as it has been shown that cancers that become resistant to MDM2 inhibitors often amplify *MDM4*. Finally, we will also touch on gene therapy and vaccination approaches; the former of which aims to replace mutated *TP53* and the latter whose goal is to activate the body's immune system toward mutant p53 expressing cells. Besides the obvious importance of MDM2 and MDMX expression for regulation of p53, other regulatory factors should not be underestimated and are also described. Despite the beauty of the concept, the past years have shown that many obstacles have to be overcome to bring p53 reactivation to the clinic on a broad scale, and it is likely that in most cases it will be part of a combined therapeutic approach. However, improving current p53 targeted molecules and finding the best therapy partners will clearly impact the future of cancer therapy.

280 words 300 allowed

Keywords: p53, MDM2, MDMX, *TP53* mutation, *TP53* gain-of-function, gene therapy

Introduction

The tumor suppressor protein p53, also called “the guardian of the genome”, protects the genome by responding to cellular stress and DNA damage; promoting transient cell cycle arrest enabling DNA repair, an irreversible cell cycle arrest called senescence or cell death depending on the type of stress and the cellular environment (Vogelstein, Lane, & Levine, 2000). It therefore follows that the p53 protein-coding gene *TP53* is the most frequently mutated gene in human cancers mostly through point mutations in exons 5-10, whereby amino acid substitutions lead to disruption of p53 binding to DNA and subsequent loss of function (LOF). Hence, cells in the process of transformation harboring mutant *TP53* can develop an unstable genome, evade apoptosis and may finally develop into malignancies. Therefore, it is not surprising that *TP53* mutated or deleted tumor cells are resistant to many chemotherapeutic agents since the basis of their efficacy is DNA damage leading to activation of intact p53. As such, mutated *TP53* is found in over 50% of all human cancers but the incidence of p53 mutatants varies significantly between cancer types, ranging from 95% in uterine carcinomas to 5% in cervical or thyroid cancer (www.cbioportal.org). However, there is also growing evidence that some mutant forms of p53 lead not only to loss of wild-type (WT) p53 tumor suppressive function but also display gain of function (GOF) properties that infer a survival advantage during tumor evolution (G. Liu et al., 2000; Xiong et al., 2014). For example, mice lacking expression of p53 as a result of gene ‘knock-out’ show a different disease phenotype to mutant p53 ‘knock-in’ mice whereby the presence of mutant p53 enhances tumor metastasis (G. Liu et al., 2000; Xiong et al., 2014). Consequently, not all mutations detected in p53, in tumor cells are equal in their

consequences to p53 function; as mentioned above, some mutations lead to LOF, others to GOF and some act in a dominant-negative manner over the WT protein when present in heterozygosity.

Moreover, p53 is highly regulated by an intricate network of modifier proteins enabling or inhibiting p53 functions. These interdependent signaling pathways provide many, sometimes unexpected, targets for therapeutic intervention. Central to the function of p53 is its transcription factor activity whereby it binds as a tetramer to DNA at the repetitive consensus binding site sequence (CATGXXXXXCATG) (Cui, Kerby, McDuff, Ye, & Turner, 2009; Nikulenkov et al., 2012). Currently, more than a hundred target genes have been described (Riley, Sontag, Chen, & Levine, 2008) but the most studied include pro-apoptotic BH3 only proteins (e.g. Puma, Bax, Noxa), the cell cycle inhibitor p21, death receptors (e.g. Fas) and non-coding RNAs like miR-34a (Asslaber et al., 2010). Regulation of p53 activity under physiological conditions is largely controlled by proteosomal-mediated degradation following ubiquitination at the C-terminus by a network of negative regulatory proteins such as murine double minute 2 (MDM2) and MDMX (encoded by the *MDM4* gene) (Moll & Petrenko, 2003). The E3 ubiquitin-ligase MDM2 triggers proteosomal degradation while MDMX, which has no ubiquitin ligase function (due to a dysfunctional RING domain), forms heterodimers with MDM2, thereby enhancing its activity (Linares, Hengstermann, Ciechanover, Muller, & Scheffner, 2003). As such, both *MDM2* and *MDM4* are considered oncogenes and their over-expression is seen in cancers such as sarcomas (20%) and breast cancer (15%). Function and stability of p53 is also regulated by diverse post-translational modifications largely in response to stress signals: for example, phosphorylation by the

DNA damage-induced kinases ATM/ATR and checkpoint control kinases CHK1/CHK2 upon genotoxic stress at the N-terminal domain (reversed by phosphatases like PPA2), acetylation at the negatively charged C-terminus and the DNA binding domain by the histone acetyl transferase P300/CBP and methylation of the C-terminus by the methyltransferases SET9 and/or PRMT5 (Figure 1). Conversely, the NAD-dependent histone deacetylase SIRT1 attenuates p53 function by deacetylation (Figure 1). It is important to consider that similar to epigenetic marks on histone tails, methylation, acetylation or ubiquitination can even affect the same lysine residue within p53, often leading to opposing biological effects.

Thus, different strategies are needed for therapeutic approaches towards *TP53* mutant and *TP53* wild-type cancers. In murine models, reconfiguration of mutant p53 to its active WT function restores apoptosis and promotes retardation and even regression of advanced tumors (Martins, Brown-Swigart, & Evan, 2006; Ventura et al., 2007). In the context of *TP53* wild-type, we have shown in a $PTEN^{-/-}$ prostate cancer mouse model that disrupting STAT3 in the prostate epithelium leads to a defect in senescence control through the p14(ARF)/TP53 axis and aggressive tumor growth ensues. Hence, senescence must be overcome for cancer development and aggressive tumor growth is promoted by pro-inflammatory JAK-STAT pathway activation (Pencik et al., 2016; Pencik et al., 2015).

This review focuses on therapeutic methods to reactivate wild-type or mutant p53 and their outcome in preclinical and clinical studies.

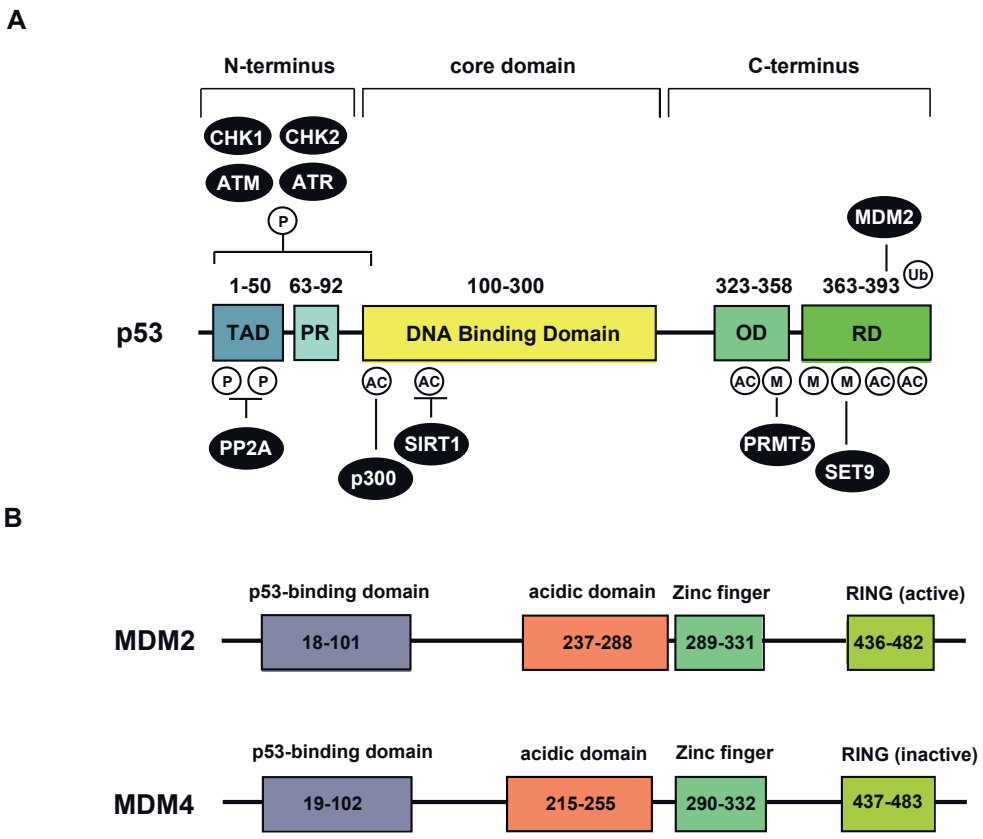

Figure 1: Schematic representation of the domain structures of p53, MDM2 and MDM4. A p53 domain structure is shown with an N-terminal transactivation domain (TAD) a proline-rich domain (PR), a DNA binding core domain (DBD), an oligomerization domain (OD) allowing for TP53 tetramerization and a protein stability regulatory domain (RD) at the C-terminus. Following DNA damage CHK1, CHK2, ATM and ATX kinases phosphorylate p53 at multiple serine and threonine residues leading to p53 stabilization, whereas the phosphatase PP2A can reverses p53 phosphorylation. The NAD-dependent deacetylase SIRT1 attenuates p53 function but p53 acetylation by P300/CBP activates it. SET and PRMT5 are methyl-(M) transferases that act C-terminally on the oligomerization and regulatory domains of p53. **B** MDM2 is an ubiquitin (Ub) ligase that acts on the C-terminus of p53. MDM2 and MDM4 (also called MDMX) have a similar domain structure with a p53 binding domain at the N-terminus, an acidic domain, a zinc finger motif and finally the RING domain at the C-terminus, which is responsible for ubiquitination (the RING domain is not active in MDMX).

1. Targeting Mutant Forms of p53

1.1 Small Molecules Targeting Mutant p53

Small molecules have been developed that specifically target mutant forms of p53 restoring p53 transcriptional activity, thereby leading to cell cycle arrest or apoptosis of tumor cells (for overview see Table 1,2). According to the International Agency for Research on Cancer's (IARC) *TP53* mutation database (April 2016), the three most often mutated residues of p53 in cancer are the arginines in positions 175, 248, and 273 (Figure 2), (Bouaoun et al., 2016). In 2002, the low molecular weight compound PRIMA-1 was identified from a National Cancer Institute (NCI) substance library screen (Bykov et al., 2002). A further compound identified by the same group, MIRA-1 was not further followed up due to toxicity issues (Bou-Hanna et al., 2015; Bykov et al., 2005). The selection criteria used by the Bykov group to identify these compounds was the ability to preferentially kill mutant p53 as opposed to *TP53* deleted cell lines by restoring transcriptional activity of mutant or unfolded p53 (Bykov et al., 2002).

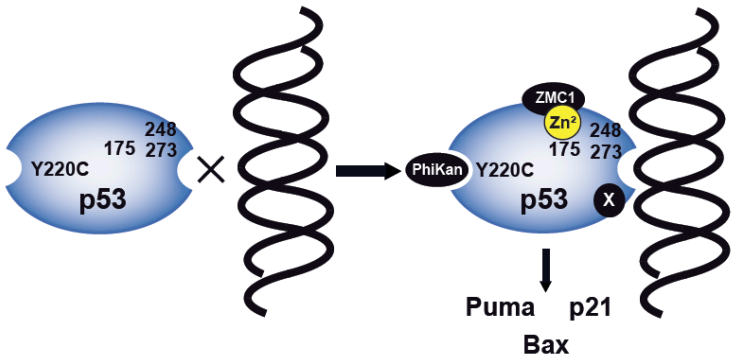

Figure 2: p53 mutations that disrupt DNA binding or destabilize protein folding and substances that can rescue these mutations. Mutations at sites 248 and 273 are the most frequently detected in cancer and are involved in direct binding to DNA. Small molecules that restore DNA binding (collectively denoted by X in the diagram) are not mutation site-specific and include PRIMA, APR-246, CP31398, Ellipticine analogs and JNJ26854165. In contrast the arginine175 mutation is important for zinc binding to p53, which is required for correct folding. This mutation can be rescued by the novel drug zinc metallochaperone 1 (ZMC-1). The Y220C mutation is not involved in DNA binding, but destabilizes p53's 3-D structure by forming a crevice that can be targeted by PhiKan-type small molecules.

PRIMA-1 and its derivative PRIMA-1^{MET}, now called APR-246, can restore a WT conformation to mutant p53 and in doing so reinstate transcriptional activity leading to expression of Puma, Noxa and Bax in p53 mutant cells (Shen, Vakifahmetoglu, Stridh, Zhivotovsky, & Wiman, 2008; T. Wang, Lee, Rehman, & Daoud, 2007). Both compounds are converted intracellularly to Michael acceptor methylene quinuclidinone (MQ) that binds covalently to cysteines in mutant p53. Moreover, transfer of MQ-modified mutant p53 into p53 null tumors induces expression of p53 downstream target genes indicating that covalent binding alone of MQ is sufficient (Lambert et al., 2009).

In vivo, PRIMA-1 has shown impressive activity in murine xenograft models of osteosarcoma (Bykov et al., 2002). Further cytotoxic and apoptotic effects of the derivative APR-246 have been observed in diverse murine cancer models such as mutant p53 small cell lung carcinoma (Zandi et al., 2011), multiple myeloma (Saha, Jiang, Yang, Reece, & Chang, 2013), melanoma (Bao et al., 2011) and breast cancer (Liang, Besch-Williford, & Hyder, 2009). In 2012, APR-246 was tested in a phase I/IIa clinical trial of 22 patients with hematologic malignancies or prostate cancer (*TP53* mutation status was not a pre-selection criterion). The drug was generally well tolerated with only transient side effects such as fatigue, dizziness, headache, and confusion. Two minor responses were observed in this late stage cohort, one in an acute myeloid leukemia (AML) patient carrying V173M mutant p53, the other one in a non-Hodgkin lymphoma patient with a *TP53* splice site mutation (Lehmann et al., 2012). Currently, APR-246 is under study in a phase Ib/II clinical trial of high grade serous ovarian cancer in combination with pegylated doxorubicin and carboplatin (NCT02098343; www.clinicaltrials.gov).

The activity of APR-246 is not limited to p53 in that it is also able to restore function of the analogous p63 mutant, thereby reversing the differentiation block caused by this mutant protein and extending the use of this exciting compound to mutant p53 analogs (Rokaeus et al., 2010). *TP53* shares high sequence homology with *TP63* in its DNA binding domains. Deactivation of p63 as a consequence of point mutations in the encoding gene leads to severe developmental defects including ectodermal dysplasia, limb defects, and orofacial clefting (EEC syndrome). APR-246 rescues epidermal differentiation in skin keratinocytes derived from patients with EEC syndrome (Shalom-Feuerstein et al., 2013; Shen et al., 2013).

Y220C p53 is one of the less frequently observed mutations, although it is estimated that 75,000 new cancer cases per year bear this mutation, which is comparable to the number of patients carrying the BCR-ABL translocation. This mutation, in the core domain of p53 is not involved in DNA binding, but the Y220C substitution creates a cavity that destabilizes p53. The small molecules PhiKan083 and PhiKan7088 bind to this cavity and therefore interact with Y220C p53. They were designed by molecular modeling based on the X-ray crystal structure of this mutant. *In vitro*, PhiKan7088 permits the correct folding of Y220C p53 restoring transactivation potential and hence inducing expression of p21 and Noxa, leading to cell cycle arrest and apoptosis (Figure 2) (Boeckler et al., 2008; X. Liu et al., 2013). Altogether, small molecular weight compounds constitute a promising approach for targeting specific mutant p53 forms and their further development addresses a great clinical need.

The quinazoline derivative CP-31398 was selected from a screen of over 100,000 compounds looking for those that could restore wild-type conformation of DNA

binding domain (DBD) mutants of p53, using the conformation-specific antibody PAb1620 as a probe (Foster, Coffey, Morin, & Rastinejad, 1999). The exact mechanism of action of CP-31398 is unclear, since NMR studies have not been able to show direct binding of CP-31398 to p53 (Rippin et al., 2002). *In vivo* studies of a transgenic APC^{min} colon cancer mouse model demonstrated that CP-31398 suppresses APC^{min} induced colon cancer development; CP-31398 treatment induced apoptosis of tumor cells with elevated levels of p53 and expression of p21 detected (Rao, Swamy, Patlolla, & Kopelovich, 2008). Furthermore, in a mouse model of UVB-induced skin carcinogenesis, a reduction in tumor growth was observed (Tang et al., 2007). More recently, when applied to pancreatic adenocarcinoma cell lines, CP-31398 induced apoptosis as well as autophagy, although active concentrations were above 10μM (Fiorini et al., 2013). New analogs have now been developed that lack the unstable styryl linkage in the side chain of CP-31398 and are showing promising first results (Sutherland et al., 2012). Another small molecule that specifically binds the p53 DBD is SCH529074; this interaction restores wild-type function and concomitantly inhibits ubiquitination of p53 by HDM2 (Demma et al., 2010).

Ellipticine analogs have also been tested for p53-dependent cytotoxic activity within the anticancer drug discovery program of the US National Cancer Institute (NCI) (Shi et al., 1998). Ellipticine restores transactivation inducing expression of p21 and MDM2 in several p53 mutant-expressing cell lines as well as in nude mouse tumor xenografts (Peng, Li, Chen, Sebti, & Chen, 2003). Ellipticine was shown to sensitize p53 mutant cells to doxorubicin-induced apoptosis in human lymphoma cell lines (F. Wang et al., 2011). However, other mechanisms such as DNA intercalation,

topoisomerase II inhibition and formation of DNA adducts may also be involved (Poljakova et al., 2009).

Similar to PRIMA discussed above, RITA (reactivation of tumor cell apoptosis) was also derived from the NCI library. RITA is able to kills p53 WT HCT116 colon cancer cells more effectively than the isogenic p53 mutant version of this cell line and also shows activity in murine xenograft models (Issaeva et al., 2004; J. Yang et al., 2009). RITA targets the N-terminus of p53 and through conformational change prevents the binding of negative regulators such as MDM2 or HPV E6 (Enge et al., 2009; C. Y. Zhao, Szekely, Bao, & Selivanova, 2010). More recently, Selivanova and colleagues showed in neuroblastoma that RITA's mechanism of action can be extended to both mutant (C135F, M246R) and WT p53. When activated by RITA, p53 inhibited key oncogenes including n-Myc, BCL2, MDM2 and MDMX (Burmakin, Shi, Hedstrom, Kogner, & Selivanova, 2013). Recently, a study suggested that RITA-resistant cell lines show increased DNA cross-link repair and are therefore also resistant to crosslinking agents like cisplatin, indicating that the mechanism of action may be inducing DNA damage upstream of p53 rather than a direct effect on p53 (Wanzel et al., 2016).

1.2 Restoration of Mutant p53 with Zinc

p53 contains a single zinc ion close to its DNA binding interface. Zn^{2+} is essential for site-specific DNA binding, activation of downstream targets and correct protein folding. Loss of metallothioneins, which store intracellular zinc, promotes the unfolding of WT p53 and inhibits its transcriptional activity (Puca et al., 2009). If p53 bears the R175H mutation its capacity for zinc binding is abrogated.

Screening of a small compound library has shown that the small molecule zinc metallochaperone-1 (ZMC1) was able to restore WT function specifically to this mutant form of p53. The reason for this may be that ZMC1 is a zinc ion chelator, which enables transport of the ion through the cell membrane (Blanden et al., 2015; X. Yu et al., 2014). ZMC1 may pave the way for future clinical molecules that restore function to the common R175H p53 mutant. Another compound NSC319726 seems also to be a zinc-chelator and restores function of the R175H p53 mutant in both cancer cell lines and tumor xenografts (X. Yu, Vazquez, Levine, & Carpizo, 2012).

1.3 Proteosomal Depletion of Mutant p53

Rationale for this strategy includes selective degradation of dominant negative mutant forms of p53 or the depletion of mutant forms that have GOF properties. For this approach, compounds were screened for degradation of mutant p53 but not WT p53. Mutant p53 is stabilized by interaction with the heat shock protein Hsp 70 and Hsp 90 chaperone complex that depends on HDAC6 interaction for activation (Li, Marchenko, Schulz, et al., 2011). Several Hsp 90 inhibitors have been developed and include geldanamycin, 17-AAG and ganetespib. In murine models, ganetespib showed a 50-fold higher potency than 17-AAG in destabilizing mutant p53 (Alexandrova et al., 2015). Ganetespib was tested in a phase II trial for Non-Small Cell Lung Cancer (NSCLC) together with docectaxel and showed increased progression-free survival although a phase 3 study that closed this year (NCT01798485) could not reproduce these findings (Ramalingam et al., 2015).

Similar approaches include, histone deacetylase inhibitors (HDAC) such as suberoylanilide hydroxamic acid (SAHA), also called Vorinostat or Zolinza. Vorinostat can destabilize the complex formed between Hsp90 and mutant p53 and shows preferential cytotoxicity in mutant p53 cancer cells (Li, Marchenko, & Moll, 2011). Vorinostat was approved by the FDA in 2006 for the treatment of cutaneous T cell lymphoma and is being trialed in other cancers including myelodysplastic syndrome and Sézary syndrome (L. Ding et al., 2015).

Arsenic trioxide (ATO) is used in combination with all trans retinoic acid (ATRA) as a curative therapy for acute promyelocytic leukemia (APL). Like ATRA it also leads to the degradation of the PML-RARα fusion protein thereby allowing terminal cell differentiation (Lallemand-Breitenbach & de The, 2013). We have shown that p53 mutant cells of chronic lymphocytic leukemia patients are selectively killed by ATO (Merkel et al., 2008). This may at least in part be explained by data from Yan and colleagues who showed that ATO can reactivate proteasome-dependent degradation of mutant p53 in cancer cells partly through enhanced expression of the Pirh2 E3 ligase (Yan, Jung, Zhang, & Chen, 2014).

Disulfiram (DSF) has ubiquitin proteasome-targeting activity inhibiting acetaldehyde dehydrogenase and as such is in use for the treatment of chronic alcoholism. Interestingly, DSF was shown to act synergistically with doxorubicin in the mutant p53 breast cancer cell line MDA-MB-231(Robinson et al., 2013). Recently it has been evaluated as an adjunct to chemotherapy in metastatic NSCLC with remarkable success (Nechushtan et al., 2015). Gambogic acid is derived from the Garcinia hanburyi tree and induces proteasomal degradation of mutant p53 (J. Wang et al.,

2011). Spautin-1, an inhibitor of autophagy also has an unexpected role in mutant p53 degradation. When cancer cells are challenged by starvation, cells start a process of macro-autophagy during which intracellular proteins and organelles are degraded through lysosomes, providing an alternative energy source. Spautin-1 controls levels of p53 by regulating the deubiquitinating activity of USP 10 and USP 13 (J. Liu et al., 2011). Under a glucose-free environment with confluent growth conditions, spautin-1 inhibits macro-autophagy and induces degradation of several mutant p53 forms by a specific form of autophagy, namely chaperone-mediated autophagy (Vakifahmetoglu-Norberg et al., 2013). This is an interesting new concept that may lead to novel forms of therapy for p53 mutant solid cancers and the recent Nobel prize in medicine awarded for the discovery of autophagy might raise research interest in this underexplored area.

1.4 Targeting proteins that modulate p53 function

An alternative approach towards targeting mutant p53 activity is either to reactivate tumor suppressive pathways inhibited, or to suppress oncogenic targets activated by it. As seen before, mutant p53 can exert GOF activity by binding and thereby inhibiting the tumor suppressor functions of p63 and p73. Accordingly, the disruption of mutant p53 in complex with p63 and p73 appears an alternative to the refolding approach of mutant p53. For example, the small molecule RETRA suppresses mutant p53-bearing cancer cells through inhibition of the mutant p53/p73 complex and induction of p73 expression (Kravchenko et al., 2008).

The R280K or R273H DBD mutants of p53 when engineered into mice, disrupt mammary tissue architecture by inducing the mevalonate/cholesterol synthesis pathway

(Freed-Pastor et al., 2012). Statins inhibit the rate limiting enzyme in this pathway 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely used for the treatment of hypercholesterinemia and prevention of cardiovascular disease. Hence, statins restore morphology and decrease survival of mutant p53 breast cancer cell lines (Freed-Pastor et al., 2012). Statins also block phosphorylation of oncogenic MYC, which together with their impact on cholesterol metabolism and membrane integrity could help to explain their broader anti-cancer action.

Modification of p53 is also conducted by proteins typically associated with epigenetic histone marks such as those that mediate lysine methylation and acetylation. For example, the methyltransferases SMYD and SET7 methylate lysines in the C-terminus of WT p53 in teratocarcinoma cell lines compromising its transcriptional activity. Thus, inhibition of these proteins leads to p53 reactivation and differentiation of the teratocarcinoma cells suggestive of a possible therapeutic avenue (Zhu, Dou, Sammons, Levine, & Berger, 2016). In a similar vein, mutant GOF p53 has been shown to bind to the transcription factor ETS2 thereby activating transcription of the methyltransferases MLL1 and MLL2 as well as the acetyl transferase MOZ1. MLL family members are core components of large protein complexes that modify chromatin structure and hence gene transcription and in the case of p53 GOF mutants, induce transcription of genes associated with tumor cell proliferation. Thus, highly specific inhibitors of MLL complex formation in p53 GOF mutant cells block cell proliferation but do not affect p53 WT cells (Zhu et al., 2015).

2. Reactivation of WT p53

2.1 MDM2 and/or MDMX Inhibitors

In numerous tumors, *TP53* is not mutated but inactivated due to (over)expression of negative regulatory proteins such as MDM2 and MDMX. Deregulation of the autoregulatory feedback loop between p53 and MDM2 proteins neutralizes p53 tumor suppressor function. Therefore, disruption of p53/MDM2/MDMX interactions by small molecules may reactivate the p53 pathway in tumor cells retaining WT p53. The MDM2 protein was isolated in 1987 as the protein product of extrachromosomal circular DNA amplifications, also called 'double minutes', in transformed 3T3 cell lines (Cahilly-Snyder, Yang-Feng, Francke, & George, 1987). MDM2 regulates p53 by two main mechanisms: inducing p53 degradation (by acting as an E3-ubiquitin ligase) and directly suppressing the transactivation domain of p53, hence preventing transcription of downstream targets. In contrast, MDMX has no ubiquitin ligase function, but shares MDM2s ability to inhibit transcription of p53 downstream targets. A prerequisite for the ubiquitin ligase function of MDM2 is the formation of homo-oligomers, which depends on the carboxy-terminal RING domain; MDMX does not form homo-oligomers. However, hetero-oligomerization between MDMX and MDM2 is needed for optimal ubiquitin-ligation through MDM2 (Francoz et al., 2006; Momand, Zambetti, Olson, George, & Levine, 1992; Wu, Bayle, Olson, & Levine, 1993). The p53/MDM2 interaction brings together the N-terminus of the p53 transactivation domain with the first 120 amino acids of the MDM2 N-terminus. The X-ray crystal structure of MDM2 bound to a p53 peptide revealed Phe19, Trp23 and Leu26 as the key residues creating a locally restricted, hydrophobic interaction site within p53 (see Figure 3) (Kussie et al., 1996). Accordingly, peptides with the same or similar sequence were screened for their

ability to interfere with MDM/p53 binding. Peptides are polar, large molecular weight compounds; structural mimetics and screens of compounds blocking MDM2 binding are an area of future investigation.

2.1.1 MDM2 inhibitors: peptides.

One mechanism to inhibit MDM2 activity is to design peptide-mimics of p53 that block, via steric hindrance the p53 binding site of MDM2. WT p53 peptide encompassing the helical MDM2 binding site has comparatively low binding affinity for MDM2. To improve affinity, the amino acid sequence of p53 peptides has been optimized using phage display techniques and *in silico* structure-guided approaches. Introduction of non-natural amino acids that allow for additional interpeptidic bonds have been introduced into the peptide sequence, a method also called “stapling”. This has led to enhanced affinity by reducing conformational flexibility, increasing the half live of the peptides and enabling cellular uptake. One of the first inhibitors of this type is SAH-p53-8, a stapled p53 peptide made cell permeable by the introduction of positively charged amino-acids. In contrast to an inactive mutant form, SAH-p53-8F19A, SAH-p53-8 can reactivate p53, induce p21 and lead to apoptosis of the SJSA-1 osteosarcoma cell line. However, concentrations (10-15 μM) needed to exert these effects are relatively high (Bernal, Tyler, Korsmeyer, Walensky, & Verdine, 2007). Another peptide that binds MDM2 is sMTide-02A detected using phage-display techniques and modified (through stapling, removal of proline 12 and a change of asparagine 8 to alanine) to bind to the p53-binding pocket of MDM2 with high affinity (it has a remarkably low Kd value of 6.76 nM) (Pazgier et al., 2009; Phan et al., 2010).

This peptide leads to G1 and G2 arrest in different p53 wild-type cell lines without induction of apoptosis (Brown et al., 2013).

2.1.2 MDM2 inhibitors: small molecules.

The small size of the MDM2/p53 interface has made it possible to design not only peptides but also small organic compounds that block this interaction (Figure 3). Compound screens, structure-guided *in silico* searches or *de novo* design, have identified molecules that are able to disrupt this interaction by binding the MDM2/p53 interface of MDM2. The most promising MDM2/p53 disruptors belong to different classes of compounds: nutlins, spirooxindoles and piperidinones. Nutlin-3a was developed in 2004 by Hoffmann la Roche with the group of Vassilev et al. (Vassilev et al., 2004) and inhibits the association of MDM2 with p53 via binding to the hydrophobic cleft of MDM2 (Figure 2 B). Nutlin-3a is a potent MDM2 inhibitor and shows efficacy in primary chronic lymphocytic leukemia cells that are wild-type for p53 (Coll-Mulet et al., 2006; Kojima et al., 2006; Secchiero et al., 2006) and in many tumor cell lines from various malignancies including acute myeloid leukemia (Kojima et al., 2005), chemoresistant neuroblastoma (Van Maerken et al., 2009), anaplastic large cell lymphoma (Y. Cui et al., 2009; Y. X. Cui et al., 2009) or more recently multiple myeloma (Surget et al., 2012). However, due to poor bioavailability and high toxicity its use in the clinic has been hampered. The nutlin analog RG7112 was first clinically evaluated in MDM2-amplified liposarcoma patients showing that it could indeed amplify the p53 pathway in patients (Ray-Coquard et al., 2012). In addition, a phase I clinical trial of patients with relapsed refractory leukemia was performed recently (Andreeff et al.,

2016). Toxicities include gastrointestinal and hematologic side-effects (neutropenia, thrombocytopenia). Among the AML patients, five out of 30 patients reached complete or partial responses and 9 patients entered stable disease. Furthermore, two patients with mutant p53 (R181L and G266E) showed a clinical response. The latest addition to this group of compounds is RG-7388 which shows greater effects in the induction of p53 in the osteosarcoma cell line SJSA-1 and accordingly is also able to reduce growth of engrafted SJSA-1 cells in mice at lower concentrations than RG-7112 and Nutlin-3a (Q. Ding et al., 2013). Currently, a number of phase I clinical trials with MDM2 inhibitors in combination with other agents are being evaluated for example a phase Ib study in AML patients with cytarabine (Martinelli et al., 2013) or in combination with trabectedin in sarcomas (Obrador-Hevia et al., 2015).

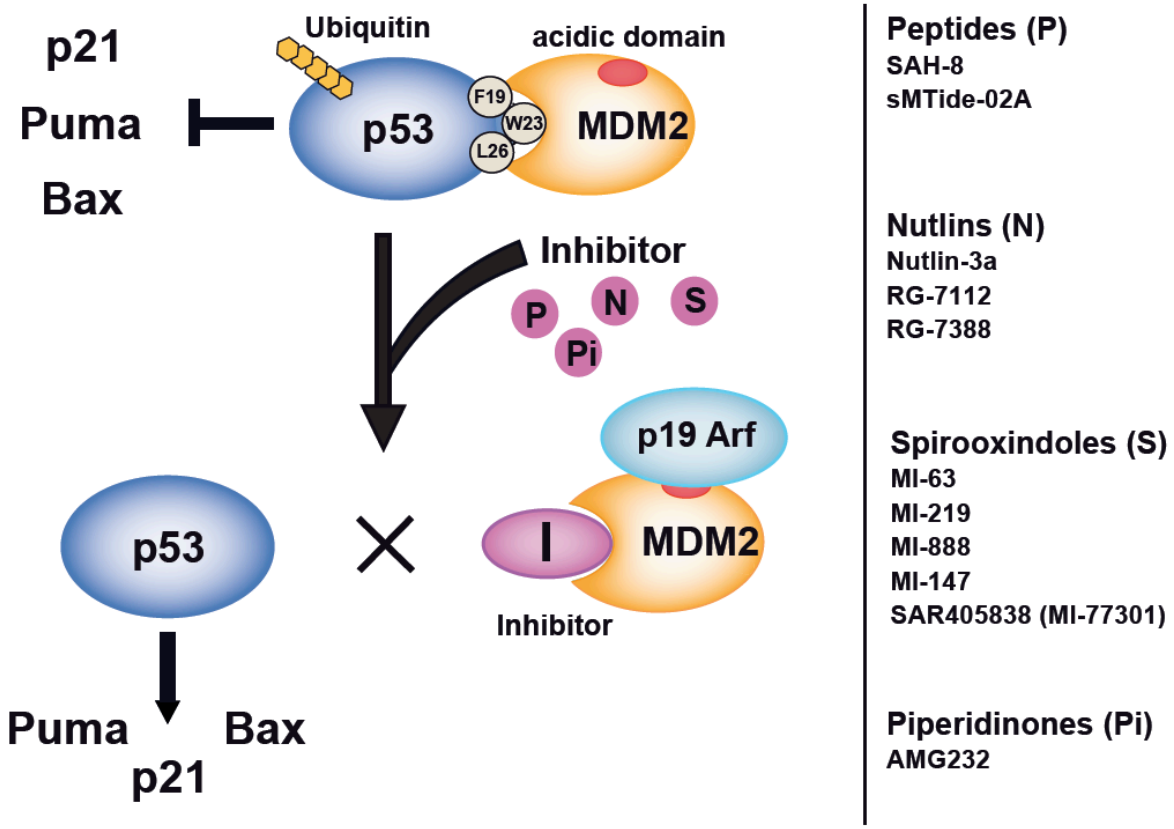

Figure 3: Drugs that interact with the MDM2/p53 binding pocket. The mechanism of most MDM2 inhibitors is binding into the hydrophobic p53-binding cleft of MDM2. Critical for MDM2 binding are the hydrophobic p53 amino acids phenylalanine 19 (F19), tryptophan 23 (W23) and leucine 26 (L26). Drug classes used to displace p53 are peptides (P), Nutlins (N), Spirooxindoles (S) and Piperidinones (Pi).

Another interesting class of MDM2 inhibitors is the spirooxindole family, initially developed by the Wang group at the University of Michigan (Y. Zhao et al., 2013). This family of compounds was identified by structure-guided *de novo* design in an effort to mimic within an organic molecule the three key hydrophobic residues in the MDM2 binding pocket of p53: Phe19, Trp23 and Leu26. This study resulted in the development of a series of 'MI' compounds, key amongst which is MI-63 which binds MDM2 with a Ki of 3 nM and in turn activates p53, inhibiting cell growth in p53 wild-type cells (K. Ding et al., 2006). Further investigations led to the development MI-147 showing p53 modulating activity in SJSA-1 osteosarcoma and HCT116 colon carcinoma cell lines (S. Yu et al., 2009). In SJSA-1 osteosarcoma xenografts, MI-147 alone and in combination with irinotecan showed strong potency and continues to undergo preclinical evaluation. Another member to this class of compounds is MI-888, which through changes in the stereochemistry reduces the Ki value to 0.44 nM and has also been shown to have activity in murine xenograft models upon oral administration (Y. Zhao et al., 2013). Finally, SAR405838, a highly potent and selective MDM2 inhibitor was identified and shown to halt tumor growth or lead to durable remissions in murine engraftment models of osteosarcoma, acute leukemia, prostate cancer and colon cancer (S. Wang et al., 2014). In dedifferentiated liposarcoma, a disease which is characterized by high MDM2 expression and usually wild type p53, SAR405838 gave rise to p53 activation and tumor regression at much lower concentrations than

comparable substances such a Nutlin3a and MI-219 (Bill et al., 2016). However, a recent publication has shown development of p53 mutations during SAR405838 treatment in patients with dedifferentiated liposarcoma, suggesting fast acquisition of resistance (Jung et al., 2016).

Another class of MDM2 inhibitors are piperidinones, such as AMG232 that inhibits tumor growth in SJSA-1 osteosarcoma xenografted mice (Canon et al., 2015; Sun et al., 2014). Currently, AMG232 is under investigation in a phase I study of patients with solid tumors or multiple myeloma (NCT01723020), in a phase Ib study with or without co-administration of the MEK1/MEK2 inhibitor trametinibin in AML (NCT02016729) and in a phase Ib/IIa in combination with trametinib and dabrafenib in patients with metastatic melanoma (NCT02110355).

2.1.3 MDMX inhibitors.

Resistance to MDM2 antagonists can develop as a consequence of amplification of *MDM4* preventing transactivation of p53 target genes (Graves et al., 2012; Wade, Rodewald, Espinosa, & Wahl, 2008; Wade, Wong, Tang, Stommel, & Wahl, 2006). Therefore, in such cases parallel inactivation of MDMX may be of benefit. Whilst most MDM2 inhibitors also bind MDMX they do so with a lower Kd that is not sufficient for total deactivation. For example, *in vitro* binding studies have shown that Nutlin-3a binds MDM2 with 500-fold selectivity over MDMX. Hence, there is a need to develop specific MDMX inhibitors. One of the first of this class of compounds is SJ-172550, which binds reversibly to MDMX inhibiting the p53 binding domain and inducing apoptosis in retinoblastoma cells known to express high levels of MDMX (Reed et al., 2010).

However, the stability of covalent adducts formed between SJ-172550 and thiol groups within the MDMX p53-binding pocket is dependent on variable factors, which complicates its use as a therapeutic agent (Nikulenkov et al., 2012). A second compound is XI-006, which acts through suppression of MDMX transcription as assessed by a MDMX promoter-linked luciferase assay (H. Wang, Ma, Ren, Buolamwini, & Yan, 2011) whereas RO-5963 leads to forced dimerization of MDMX through interaction with its RING-domain, inhibiting its ability to form complexes with p53 and as a consequence was shown to overcome Nutlin-3a resistance when due to high expression of MDMX (Graves et al., 2012). A scheme of the mode of action of the MDMX inhibitors described is given in Figure 4.

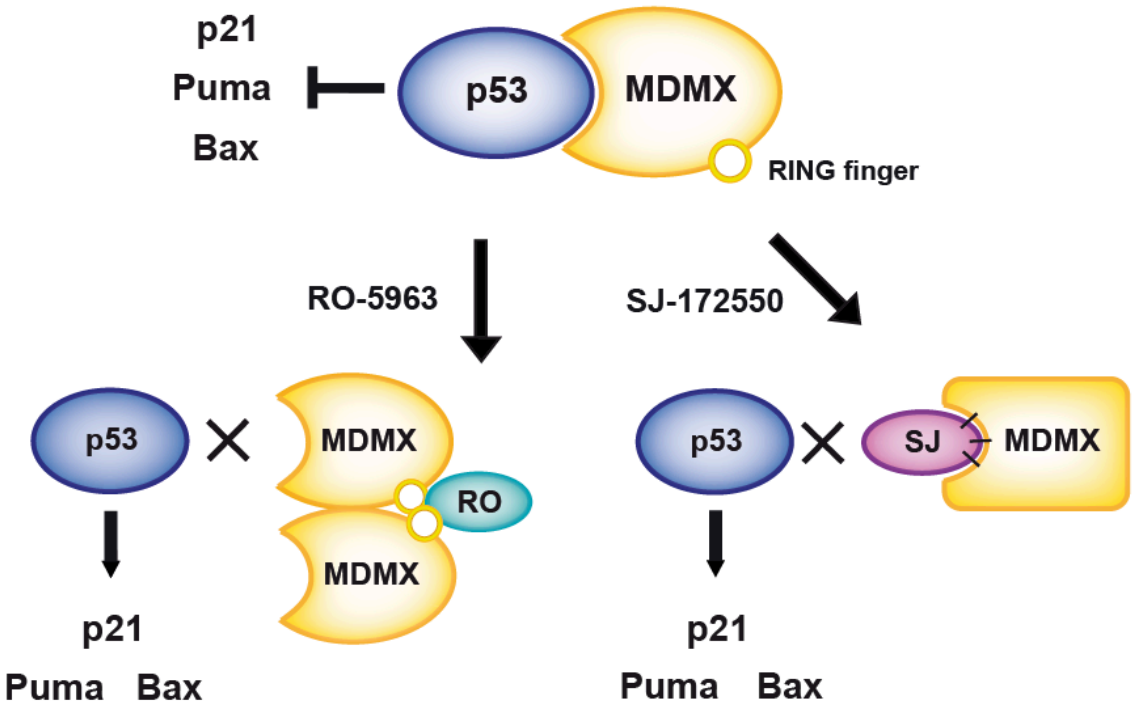

Figure 4: MDMX inhibitors and their mechanism of action. SJ172550 is a compound that binds to the p53 binding pocket of MDMX via disulfide bonds. RO-5963 acts by dimerization of MDMX through binding to the RING domain.

Another lead in the design of dual MDM inhibitors is lithocholic acid, a steroid fatty acid also naturally present in bile acid, which was found to preferentially target MDMX over MDM2, raising the possibility that certain steroids may be natural ligands of MDM proteins. However, low solubility and apoptosis inducing activity only at concentrations above 100 μM prevent its use as a therapeutic drug (Vogel et al., 2012). Lipid side chain modifications of lithocholic acid and cytochrome P450 action might overcome these limitations in future approaches.

2.2 Targeting p53 Upstream Regulators

As previously mentioned, p53 stability and activity is regulated by post-translational modifications and therefore modulation of these events also presents therapeutic options. Persistent acetylation of p53 promotes protein stabilization and also activation of downstream targets. Tenovin-6, a small molecular weight sirtuin deacetylase SirT1 enzyme inhibitor blocks p53 deacetylation. Hence Tenovin-6 induces apoptosis of acute lymphoblastic leukemia cells and their stem cells, as well as uveal melanoma cells with mutant p53 (Dai, Zhou, Jin, & Pan, 2016; Jin et al., 2015). When DNA damage induces cell cycle arrest it is no surprise that p53 mutant cells are highly dependent on the G2/M checkpoint, since they are unable to initiate G1 arrest via p21 and CDK2. Therefore it is an interesting “synthetic lethal” strategy to enforce lethal G2/M transition, by keeping the checkpoint kinase CDK1 in its active dephosphorylated state (Bauman & Chung, 2014). This can be achieved by inhibiting the kinases Wee1 (MK-1775) or Chk1 (AZD7762) that usually mediate G2/M arrest by ensuring CDK1 phosphorylation. Accordingly, in a p53 mutant head and neck squamous cell carcinoma

(HNSCC) engraftment model, the Wee1 kinase inhibitor MK-1775 displays single-agent activity and potentiates the efficacy of cisplatin (Moser et al., 2014).

3. Vaccination and Gene Therapy Approaches

3.1 *TP53* Vaccination

Tumor vaccination is an exciting concept in the era of immunotherapy and tumor neoantigen vaccination strategies yet cancer vaccination programs have gone through phases of enthusiasm and disappointment. Recently, further development and a better understanding of the immune system have led to improved novel strategies. Early phase clinical trials using peptides that activate dendritic cells or vector-based strategies have had very limited clinical effect, suggesting effective immune evasion by tumors (Goldman & DeFrancesco, 2009). Immunomodulatory escape mechanisms include changes in $CD4^{+}FoxP3^{+}$ regulatory T cells (Tregs) suppressing the adaptive immune response, expression of the co-stimulatory protein CTLA-4, which induces T-cell anergy and/or differential regulation of programmed death ligand 1 (PD-L1) and its receptor PD-1 restraining the tumor killing effect of T-cells (Chen & Han, 2015; Ma, Gilligan, Yuan, & Li, 2016; Ohaegbulam, Assal, Lazar-Molnar, Yao, & Zang, 2015).

Ideally, the tumor-associated antigen used for vaccination should have three properties: First, it should be exclusively expressed on tumor tissues, second, be essential for tumor growth and third, possess immunogenic properties. Unfortunately, p53 is expressed in both healthy and tumor cells. Nevertheless, it has been shown that CD8 T cells directed against wild-type p53 can discriminate between p53-presenting tumor cells and normal tissue (Nijman, Lambeck, van der Burg, van der Zee, &

Daemen, 2005). The reasons for this remain unclear and may be related to the higher levels of p53 expressed in cancers, particular mutant forms of p53 leading to elevated levels of processing and presentation on immune cells. Indeed, higher levels of p53 antibodies have been found in patients with lung, pancreatic, bladder and breast cancer and in patients expressing mutant forms of p53, although the levels of antibody production are obviously insufficient to eradicate the tumors (Angelopoulou, Diamandis, Sutherland, Kellen, & Bunting, 1994; Met, Balslev, Flyger, & Svane, 2011). In a study of 21 ovarian cancer patients, p53 peptide-pulsed dendritic cells (p53 amino acids 264-272) were compared to subcutaneous injection of the same p53 peptide together with the myeloid cytokine GM-CSF. An immune response was detected in 83% and 69% of the patients as tested by ELISPOT-assays in the two arms respectively, suggesting successful immunization in both arms. Administration of IL-2 in both arms generated grade 3-4 toxicities and boosted the presence of Tregs precluding its clinical applicability. Both arms of the study had similar progression-free and overall survival, with minor toxicities, suggesting subcutaneous injection to be as effective as peptide-pulsed approaches (Rahma et al., 2012). More recently, the results of a phase Ib trial dendritic cell p53 peptide vaccine for head and neck cancer were published (P. J. Schuler et al., 2014). Adjuvant p53 vaccination of 16 patients was safe with a two-year disease-free survival of 88%. In 69% of the patients, p53-specific T cells increased post-vaccination confirming the rational for the study.

3.2 Gene Therapy Strategies

TP53 gene therapy is based on delivery of a wild-type *TP53* gene into tumor cells. The first human study was reported in 1996 by Roth et al., in which a retrovirus carrying human *TP53* was directly injected into NSCLC (Roth et al., 1996). However, use of retrovirus remains a threat due to the potential for oncogene activation as a result of retroviral insertion into the host genome (Bishop, 1982). Hence, alternatives that minimize this problem have been explored including adenoviral vector gene delivery. One such vector is Gendicine, a recombinant adenoviral human *TP53* vector that received approval in China in 2003 for use in combination with radiotherapy for the treatment of head and neck cancer. Gendicine has also been investigated for its efficacy when administered in combination with fractionated stereotactic radiotherapy for the treatment of hepatocellular carcinoma and showed improved one-year survival rates (Z. X. Yang et al., 2010). In the USA, adenoviral p53 vectors such as SCH-58500 (CANJI, Inc.) and Advexin (Introgen Therapeutics) have been investigated in various clinical trials (M. Schuler et al., 2001; Shimada et al., 2006). However, companies pursuing this avenue have since closed down like Advexin, or been bought by Merck like CANJI, Inc. and no active clinical trials can currently be found on databases suggesting severe problems associated with this strategy.

3.3 *TP53* Oncolytic Viruses

Because of the low transduction efficiencies of *TP53* via adenoviral-based vectors, other strategies have been developed. This approach uses replicating viruses for selective killing of tumor cells and is based on the long-known observation that tumors regress following viral infection, which may be caused by interferon and or

acquired immune responses (Bluming & Ziegler, 1971; Hansen & Libnoch, 1978). An example of *TP53*-based oncolytic virotherapy is dl-1520 (Onyx-015) in which the viral *E1B-55KDa* gene has been deleted. E1B proteins interact with p53, inducing its degradation, therefore enabling host cell survival and subsequent viral replication. Hence, in *TP53*-deficient tumors, virus lacking the E1B proteins are able to replicate, whereas in normal cells p53 is activated preventing cellular survival and as a corollary viral replication. As such Onyx-015 presents a relatively specific therapy whereby virus replication only occurs in tumor cells that lack p53 resulting in cytolysis (Bischoff et al., 1996). Onyx-015 was investigated in numerous phase I/II clinical trials of malignant gliomas, hepatobiliary tumors and sarcomas (Chiocca et al., 2004; Galanis et al., 2005; Makower et al., 2003). However, the clinical response to Onyx-015 was mixed, it reached phase III in combination with chemotherapy for head and neck cancer, but the trial was not completed and Onyx-015 did not obtain FDA approval. For different reasons, further clinical development was not pursued. A further oncolytic virus, H101 was developed and licensed for cancer therapy in China. H101 lacks the viral E1B genes in addition to E3 which encodes the adenovirus death protein (W. Yu & Fang, 2007). In phase II/III trials for treatment of head and neck carcinomas, it showed synergistic antitumor effects in combination with chemotherapy. Two further oncolytic viruses are currently under investigation: H103, which has completed phase I trials and H102 which is still in the preclinical phase (Farnebo, Bykov, & Wiman, 2010; Guan, He, & Zou, 2016).

3.4 MicroRNA based strategies

MicroRNAs (miRNA) are small noncoding RNAs with a typical length of around 20 nucleotides. miRNAs usually bind to the 3‘-untranslated region of their target mRNAs and can either lead to degradation of the target mRNA or block translational protein synthesis. Most miRNAs regulate multiple target genes and play an important role in physiological and pathological processes including cancer (Bartel, 2009). Recent investigations have highlighted the contribution of miRNA in p53 regulation. A group of miRNAs including miR-25, miR-30d and miR-125b have been shown to directly downregulate p53 expression whereas others including miR-192, miR-194, miR-215 and miR-605 indirectly stabilize p53 through suppressing MDM2 (Deng & Sui, 2013; Pichiorri et al., 2010). Of note, miRNA-34a, initially described as a p53 downstream target, can upregulate p53 activity through inhibition of multiple p53 negative regulators like SIRT1 (Hunten, Siemens, Kaller, & Hermeking, 2013). miRNA-34a is an important tumor suppressor and its expression is silenced by epigenetic alteration in various cancers including esophageal, gastric, hepatocellular and colorectal cancers (Hermeking, 2010). Two approaches for miRNA-34a restoration are currently under investigation: Firstly, the use of epigenetic drugs which inhibit DNA methylation and histone deacetylation and thereby restore silenced miRNA-34a expression such as a combination of 5-Aza CDR and Vorinostat in the setting of pancreatic cancer (Nalls, Tang, Rodova, Srivastava, & Shankar, 2011). Secondly, systemic delivery of miRNA-34a mimics such as MRX34 developed by Mirna Therapeutics which is currently in phase I clinical trial as a monotherapy. Using this approach, partial responses have been reported in hepatocellular carcinoma, renal cell carcinoma and acral melanoma patients although, in three patients serious adverse events that were possibly immune-

related were reported at the ASCO 2016 meeting (Beg Shaalan, 2016). Therapeutic approaches employing miRNA are still hampered by issues of poor cellular uptake and will need careful monitoring for potential side effects due to their multiple targets.

4. Outlook

To guard the integrity of a genome one has to restore the normal function of the "guardian of the genome", namely p53. There are a plethora of direct or indirect targeting approaches that all have promising and important clinical implications: from those aiming either directly at, or upstream of p53, e.g. MDM2/MDMX, to immunotherapy and vaccination strategies, to miRNA targeting. In the clinical setting it will be important to tailor the strategy to the specific tumor type and its molecular status concerning p53, MDM and other biomarkers that may eventually be unraveled.

To date, small molecule inhibitors are those holding the greatest promise. Their size, polarity, solubility, pharmacokinetic and pharmacodynamic properties can be improved through sophisticated medical chemistry approaches. Furthermore, structural modeling and drug-target protein interaction studies have revealed a detailed, atomic-level understanding of vulnerable nodes of p53 or MDM family members and how key protein interphases or pockets can be targeted. p53 cancer research activity in the future will include advanced structure-guided modeling combined with biological read-out systems. Also, *in vivo* targeting in advanced p53 animal model systems and related early phase clinical trials will bring new momentum. Studies of inhibitors of MDM2 are, good examples of how basic research into regulatory factors of p53 has enabled the development of new drugs for research and for therapeutic application. Other recently

identified non-MDM-targeting negative regulators of p53, including PIRH2, COP1 and TRIM24, also appear promising as small molecule inhibitors (Allton et al., 2009; Dornan et al., 2004; Leng et al., 2003). Assessment of the mechanisms and efficacy of these agents in murine models that overexpress PIRH2, COP1 or TRIM24 is likely to generate new insights. New and novel p53 regulators, some of them even cancer type-specific, will likely be identified in the near future from ongoing cancer genome DNA sequencing efforts and searches for mutual exclusivity with p53 aberrations. Finally, the transcriptional p53 targets HAUSP and WIP1 that stabilize MDM2 protein, thus creating a regulatory feedback loop, are natural targets for future therapeutic inhibition in cancers where one of them is amplified (e.g. invasive breast cancer or pancreatic neuroendocrine tumors; TCGA database)(Hu et al., 2010; Jiao et al., 2011). In regards to gene therapy, future work and improvement of technology e.g. CRSPR/CAS9 or the use of engineered viruses may solve the problems associated with traditional approaches (Vannucci, Lai, Chiuppesi, Ceccherini-Nelli, & Pistello, 2013). Despite the dwindling number of gene therapy trials following the dramatic setbacks caused by the development of deadly leukemia in 9 out of 9 pediatric patients after viral gene therapy for X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al., 2003), site-specific, targeted integration of virally delivered genes into the genome of patient cells is an active area of research. Newly engineered viruses derived from the lentivirus HIV and the adeno-associated virus (AAV) which belongs to the parvovirus family are potential alternatives (Gil-Farina & Schmidt, 2016). Finally, the advent of CRISPR/CAS9 technology allowing for gene insertion at defined sites in the genome

may overcome the oncogenicity observed with classic viral approaches (Cong et al., 2013).

It is remarkable how technological and scientific developments in cancer research are mirrored in the evolution of drugs targeting a single pathway: that of the p53 tumor suppressor. In the future we expect that basic research into p53 and its direct and indirect regulators will continue to generate insights that inspire academia and pharma companies to develop new, improved therapeutics for cancer patients.

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