Review

The undervalued avenue to reinstate tumor suppressor functionality of the p53 protein family for improved cancer therapy - drug repurposing.

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Abstract: p53 and p73 are critical tumor suppressors inactivated in human cancers through various mechanisms. Owing to high structural homology, the proteins share many joined functions and recognize the same set of genes involved in apoptosis and cell cycle regulation.

p53 is known as the 'guardian of the genome' and forms a critical barrier against cancer development and progression. It is mutated in more than 50% of all human cancers and the germline mutations in *TP*53 predispose to the early onset of multiple tumors in Li-Fraumeni Syndrome (LFS), the inherited cancer predisposition. Despite the ongoing effort, the treatment of cancers harbouring mutant p53 still remains challenging due to late diagnoses and the treatment-related toxicity and marginal benefit upon approval of new therapies.

Presently, the efforts focus on activating p53 exclusively, neglecting the potential of the restoration of the p73 protein in tumors. Taken that several small molecules activating wild-type p53 have failed in clinical trials, and mutant p53 reactivating drugs have not been approved yet, there is a pressing need to develop new treatments activating p53 proteins.

This review outlines the still despised therapeutic avenue, drug repurposing, which brings hope for the efficient reinstatement of the p53 protein family for improved cancer therapy.

Keywords: p53, p73, MDM2, MDMX, tumor suppressor, drug repurposing, protoporphyrin IX, verteporfin

1. Introduction

It is the media hype and the unreasonable costs of the majority of new cancer treatments, often delivering only a marginal benefit, if any, which are harming the cancer patients. More often than not, new treatments fail to deliver advancement in the outcomes, including overall survival. Surrogate endpoints applied in clinical trials usually include disease-free survival (DFS) (or progression-free survival), or overall response rates (ORR) as the primary outcome instead of a patient-centered, overall survival (OS). This fact, accompanied with the underreported financial conflicts of interest among the decisive bodies, and the biased selection criteria for clinical trial randomization, all lead to the accelerated approvals of expensive treatments which either don't or only marginally, improve the patients' outcome. As the situation looks now, it leaves little or no room for the installment of the unbiased approach in cancer care, as illustrated by Vinayak Prasad in Malignant [1].

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One way to overcome the burden of the financial toxicity in oncology stemming from the skyrocketing costs of treatments of the questionable patients' benefit, is to apply a drug repurposing approach.

Drug repurposing uses an existing drug for a different medical indication. In oncology, hard drug repurposing conveys the application of the drug from the non-oncology application, to improve the outcome of cancer therapy, often at a much lower cost than that of bringing a new treatment to the market [2]. This approach is not only economical but also takes advantage of the clinical information that is already available like pharmacokinetic and pharmacodynamic profiles, maximum tolerated dose or clinical safety which allows for shorter times for drug development [3].

The recent project by the Anticancer Fund, a not-for-profit organization, supporting clinical trials on drug repurposing for improved cancer therapy, focuses on the analysis of the promising, off-patent compounds as candidates for repositioning in oncology [4]. The REpurposing Drugs in Oncology database (ReDo) lists the drugs manifesting the anti-cancer potential and up to now includes 268 drugs (ReDO_DB) [4]. The list contains verteporfin (Visudyne®), an analog of protoporphyrin IX (PpIX), a metabolite of aminolevulinic acid showed to activate p53 and p73 [5].

p53 and its ancestor family members, p73 and p63, evolved as the cellular sensors of the DNA damage in the multicellular organisms and all proteins constitute a critical barrier against cancer development in humans. p53 is the most commonly inactivated protein in human cancers, either due to the mutations leading to the loss of wild-type p53 function or due to the overactivated oncogenic inhibitors, mainly MDM2 and/or MDMX [5].

In Li-Fraumeni Syndrome, the inherited cancer predisposition, in which *TP*53 mutations have high penetrance, the loss of p53 function drives the early onset of multiple tumors. The germline *TP*53 mutations make the treatment of LFS patients extremely challenging due to the genotoxic nature of current therapies enhancing the treatment-related toxicities, which may accelerate tumor onset in cells with mutated p53. Thus, the LFS patients are in a vast majority treated with surgery before implementing a more radical treatment. Some hopes for improved therapy of LFS with the diminished risk of secondary cancer development, are now seen with immunotherapy [6].

Taken the still tremendous cost of new therapies like the CAR-T therapy (app. 2 mln US $), there is an urgent need to develop new, more affordable, treatments for oncology patients with inactivated p53. Another challenge of cancer care stems from the treatment-related toxicity promoting a high incidence of secondary primary malignancies in cancer survivors [7].

Bearing in mind the above issues, the critical role of p53 in cancer initiation and progression, and recently reported failure of MDM2 inhibitors, RG7112, and Idasanutlin in clinical trials [8] there is an urgent new for the development of new, less toxic and affordable therapies targeting p53 proteins.

The Re_DO project outlined above collects in one site the licensed non-cancer medications as potential candidates for development of more affordable and less toxic cancer strategies. This review describes structures and tumor suppressor functions of p53 and p73 and highlights the undervalued potential of drug repurposing approach for improved cancer therapy.

2. Structure and tumor suppressor function of p53 and p73

2.1. p53

p53 is a protein of the domain structure and a transcription factor binding specifically to DNA consensus sequence consisting of two consecutive half-sites as a tetramer [9]. p53 is known to undergo multiple post-translational modifications including phosphorylation, ubiquitination, sumoylation, neddylation, acetylation, methylation, O-GlcNAcylation, ADP-ribosylation,

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hydroxylation, or recently described UFMylation [10] which are necessary for the p53 cellular turnover. In non-stressed cells, the half-life of p53 is around 20 minutes and the protein becomes stabilized and activated by the cascade of events provoked by cellular stress signals (reviewed in [11]. p53 protein stabilisation is achieved by the decreased affinity of MDM2 (or HDM2 in humans), a major p53 E3 ubiquitin ligase which drives p53 for proteasomal degradation, both in the cytosol and in the nucleus [12] [13]. The activation of the p53 transcription function occurs upon the inhibition of the binding of MDM2 to the N-terminal domain of p53 at the target DNA sequence. Since MDM2 is also a p53 target gene, a negative feedback loop exists in cells that regulates p53 activity (**Figure 1**) [12].

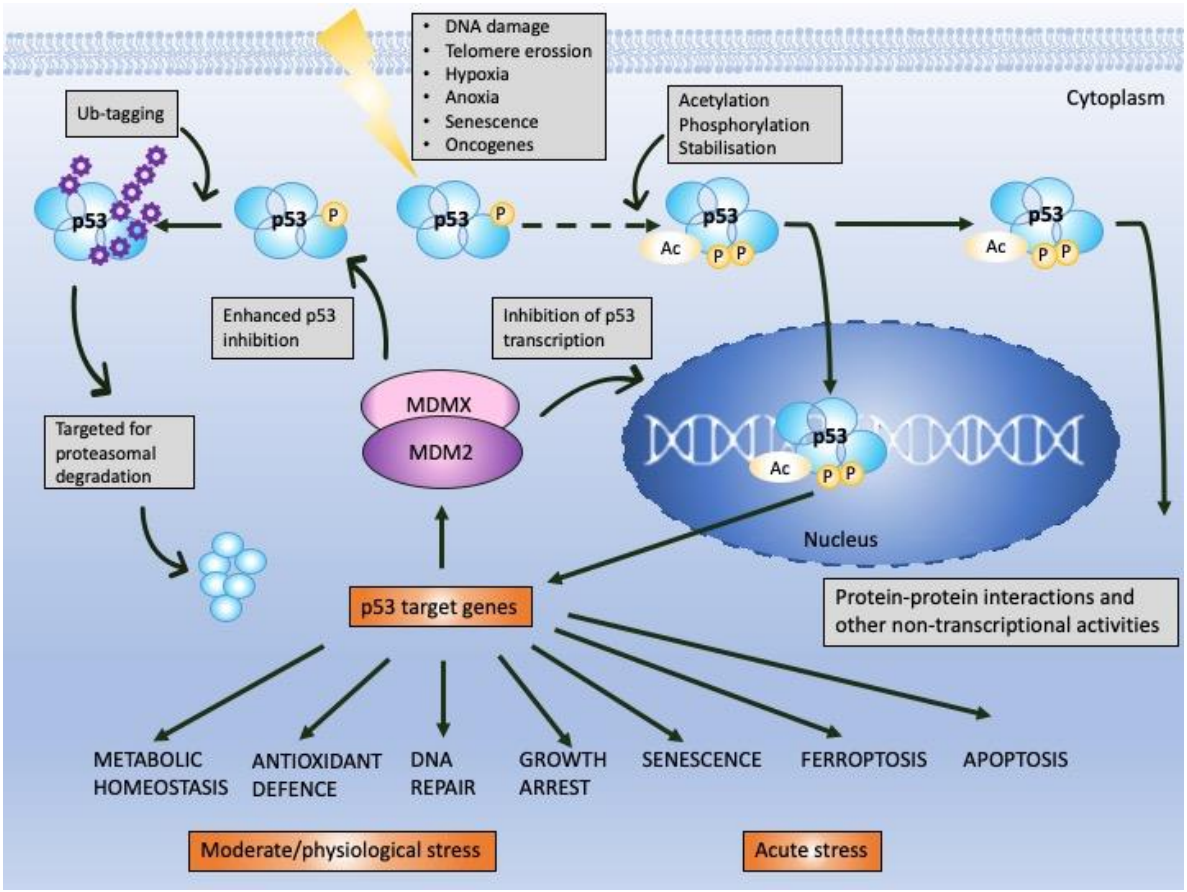

Figure 1. p53 and MDM2 as a hub of p53-dependent cellular responses – a simplified model. Under physiological conditions, p53 is degraded by MDM2, E3 ubiquitin ligase, which depending on the level of cellular stress, can have either high or low affinity to p53. MDM2 is responsible for p53 monoubiquitination (driving p53 nuclear export) and polyubiquitination of p53 (driving p53 ubiquitin-dependent proteasomal degradation) and prevents p53 acetylation and transcriptional activation by p300 acetyltransferase. The affinity of MDM2 to p53 is enhanced upon hetero-dimerization with its homolog, MDMX protein. Upon stress, p53 undergoes phosphorylation and acetylation (the sites depend on the type and severity of stress) and recognizes its target genes. MDM2 and MDMX may prevent p53 from initiating the transcription through direct binding which hinders the binding of the transcriptional co-activators. The sets of the target genes that become activated/repressed by p53 are often interrelated. In addition to transcription-dependent activity, cytoplasmic p53 functions through protein-protein interactions to modulate apoptosis, miRNA maturation or the repair of double-strand breaks (DBS). The dotted line represents a multistep process. Adapted from Levine, 2020 [14], Levine and Oren, 2009 [15] and Bode and Dong, 2004 [16].

Next, MDM2 protein, during mild stress, monoubiqutinates p53 triggering its nuclear export and enabling p53 non-transcriptional activity. MDM2 activity towards p53 is enhanced by its homolog, MDMX, which lacks the E3 ligase activity but binds to the N-terminus of p53 and alike MDM2, inhibits its transcription function [17]. Apart from MDM2 other ligases play a role in altering the p53 stability like Trim family members or Pirh as well as bacterial or viral proteins, like SV40 or E6 protein of HPV virus [18]. It is, however, the MDM2 – p53 hub that is responsible for regulating multiple cellular processes in human cells such as apoptosis, cell cycle, DNA repair, antioxidant response or senescence as well as metabolism (**Figure 1**) [14]. Further, p53 also regulates ferroptosis, iron-related cell death and has the transcription-independent function in apoptosis (binding to Bcl2-family of proteins), miRNA maturation (binding to Drosha-complex proteins) and DNA repair [19]. Its pivotal role is to orchestrate the response to genotoxic, oxidative, and oncogene-induced stress [20].

In response to mild DNA damage, activation of p53 transcription initiates cell cycle inhibition, necessary for the DNA repair to occur, and both processes converge on a cascade of protein-protein interactions (PPIs). PPIs lead to arrest in the G1 phase as p21 (CDKN1A) inhibits cyclin E2 [21] or in the G2 phase due to the blockage by the MDM2-mediated degradation of cell division cycle 25C (CDC25C) [22] and p53-mediated inhibition of CDC25C transcription [14]. The elegant study by Cuella-Martin et al., [23] showed that 53BP1 protein, a key protein involved in the repair of double strand breaks (DSB), is required for p53-induced growth arrest (co-transcriptional regulation of CDKN1A, mdm2) acting through 53BP1-dependent bivalent interactions with USP28 and p53 to intensify p53-promoter element interactions, thus enhancing p53-dependent growth arrest.

If the DNA damage cannot be repaired, the cell is directed to apoptosis, a programmed cell death. In that case, p53 transactivates BCL2 associated X, apoptosis regulator (BAX), p53 upregulated modulator of apoptosis (PUMA; also known as BBC3) and NOXA (also known as PMAIP1) [24] or interacts directly with the multidomain anti-apoptotic (Bcl-xL and Bcl-2) and proapoptotic (Bak) Bcl-2 members at mitochondria and induces mitochondrial outer membrane permeabilization and consequent cytochrome c release and apoptosis (reviewed in [25]).

p53 structure

The N-terminus domain of p53 includes transactivation domain 1 (TAD) and TAD2 (**Figure 2a, upper panel**). TAD1 and 2 work synergistically to induce transcription, and are sites of phosphorylation events leading to inhibition of MDM2 - p53 complex and to activation of p53-dependent transcription (reviewed in [26]). The X-ray structure of the MDM2 N – terminus and p53 N-terminal peptide complex shows that the minimal requirements for p53 to bind MDM2 are residues $F^{19}S^{20}D^{21}L^{22}W^{23}K^{24}L^{25}L^{26}$ [27,28]. p53 residues F19, W23 located face to face on the same side of the p53 α-helix and L26 pointing toward a cleft generated by hydrophobic pocket in the MDM2 binding site (residues L54, L57, I61, M62, Y67, V75, F86, F91, V93, I99, Y100, I103) are responsible for binding with MDM2 and MDMX [29]. Taken the well-known structure of the MDM2-p53 complex, and the fact that inhibition of the wild-type p53 (wtp53) via p53/MDM2/MDMX axis is crucial for cancer to develop (reviewed in [5]), p53 has become a very promising target for cancer therapy. Studies led to the development of rationally designed small molecules, nutlins, that bind MDM2 hydrophobic pocket with high affinity, and efficiently outcompete p53 from the MDM2 binding site [30]. The pivotal study with Nutlin-3 (IUPAC: 4-[(4S,5R)-4,5-bis(4-chlorophenyl)-2-(4-methoxy-2-propan-2-yloxyphenyl)-4,5-dihydroimidazole-1-carbonyl]piperazin-2-one), showed that it mimics the three key interactions of p53. Specifically, the imidazoline fits into the MDM2 binding site protruding three hydrophobic groups into subpockets that are normally occupied by the p53 Phe19, Trp23, and Leu26 and the piperazine ring attached to the N1 of the imidazoline is outside the binding site and does not contact MDM2. Nutlin has a much lower affinity to MDMX [31,32] and thus, it is ineffective in tumors that overexpress MDD2 and MDMX [33]. Similarly to MDM2, p53 regulates MDMX as it can bind to mRNA of MDMX and thus, regulates its translation. The p53 DBD domain binds the 5' untranslated region (UTR) of the mdmx mRNA in a zinc-dependent manner and with the trans-suppression

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domain at p53 N-terminus controls MDMX synthesis generating a negative feedback loop as observed for MDM2 [34].

The initial success of Nutlin-3 commenced the development of a series of potent MDM2-p53 inhibitors and their rapid testing in the clinical setting. Recent reports, highlighting the failure of highly specific MDM2 inhibitors like RG7112 and Idasanutlin in clinics [8] pinpoint the urgent need for the development of new therapeutic strategies aiming at wtp53 re-activation in cancer. One of the promising strategy against cancers with wild-type p53 is to apply dual inhibitors of MDM2-p53 and MDMX-p53 interactions [33]. The most advanced examples of such approach are stapled peptides, α-helical p53 stapled peptidomimetics, like ALRN-6924 peptide now in Phase I clinical development [35]. Another approach, allowing for rapid translation into clinical practice, is to apply the small-molecule dual inhibitors discovered through drug repurposing that will be discussed in detail below.

TAD domain of p53 is rendered unfolded, however, adapts transiently stable secondary structure, and the region from Phe19 to Leu22, responsible for binding to MDM2 protein, exhibits local helix propensity [36]. Due to the sensitivity to the charge-induced shifts in the structure, the otherwise liable N-terminus can be targeted pharmacologically with small molecules that alter the conformation of the protein's segment through binding. The change of conformation prevents MDM2-p53 interactions. The feasibility of such approach has already been demonstrated for a small molecule RITA, a compound that affects the interaction between p53 and MDM2 through the change of conformation of p53 N-terminus [37,38].

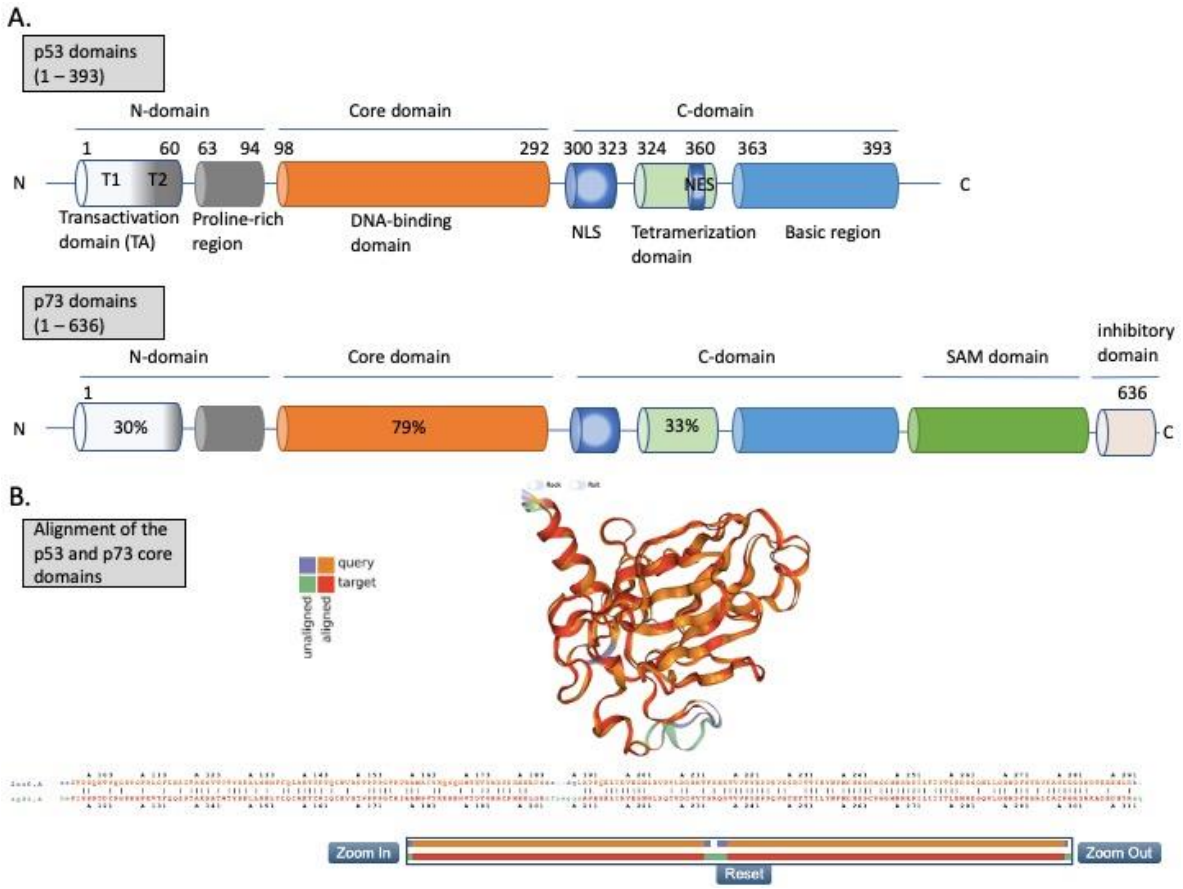

Figure 2. The structures of p53 and p73. A. *Upper panel* – domains in p53 protein. *Lower panel* – percentage homology of residues between p53 and p73 are presented and the values are indicated for each individual structural domain. T1, T2 – transactivation domain 1 and 2; NLS – nuclear localization signal; NES – nuclear export signal; SAM – sterile alpha-motif. Adapted from Tanaka et al., [39], Joanna Zawacka-Pankau et al. [40] and Melino et al., [41]. **B.** Structure alignment of p53 core domain

(PDB ID 2AC0 [9]) and p73 core domain (PDB ID 4G82 [42]) generated using Top Match Services with opacity of unmatched pairs of 0.7. https://topmatch.services.came.sbg.ac.at/index_ngl.html [43].

p53 binds specifically to its consensus sequence in the DNA through the DNA binding domain. The DNA binding domain (DBD) located centrally, spans the amino acids from 98 – 292, is preceded with the proline-rich region and two transactivation domains (TAD), TAD1 and TAD2 (**Figure 2a, upper panel**). The DBD domain is enriched in cysteine residues and contains an antiparallel β-sheet sandwich supported by loops L1, L2, and L3. Loops L2 and L3 contain amino acids for a tetrahedrally coordinated Zn^{2+} ion. The wild-type p53 protein recognizes the canonical DNA sequence motif by binding to DNA through residues K120, R273, A276, C277, R280 and R283 which bind the major groove of DNA, and S241 and R248, which bind the minor groove, and are located at the ends of these two β-sheets [44].

At the C-terminus, the regulatory basic domain is located [45]. The C-terminus is a site of multiple post-translational modifications, contains the tetramerization domain and nuclear localization signal (NLS), nuclear export signal (NES), and is involved in the interactions with DNA through non-specific DNA binding [46].

2.2. Mutant p53

Due to the ability of p53 to respond to oncogene-induced stress, TP53 is the most commonly mutated gene in cancer. More than 50% all of human cancers harbour the inactivating mutations and the six most common are the missense mutations hindering the activity of DBD domain: R175, G245, R248, R249, R273, R282 ([47,48] https://p53.iarc.fr/).

The mutations render p53 inactive and/or promote the gain of new functions to the mutated p53 protein [49]. In cases in which the TP53 gene remains intact, p53 protein is rapidly degraded by the upregulated or hyperactive MDM2 protein, E3 ligase which acts in concert with MDMX (reviewed in [5] and in [14]) (**Figure 1**). Several studies have clearly demonstrated the feasibility of targeting mutant p53 with small molecules (reviewed in [50]). The most advanced mutant p53 re-activating compound is APR-246 (eprenetapopt) discovered by Klas Wiman and colleagues [51]. APR-246 is converted to methyl quinuclidinone (MQ) and acts as Michael acceptor which targets specific cysteine residues in p53 core domain [52]. The binding of MQ to cysteine 277 increases the thermostability of the core domain in vitro and cysteine 124 and 277 are crucial for re-activation of mutp53-R175H in cancer cells. APR-246 is currently in Phase III clinical trial in combination with Azacytidine in TP53 mutated MDS and AML (reviewed in [5] and in [53]).

2.3. p73

p73 is a tumor suppressor and together with p63 and p53 is a member of the p53 protein family. p73 and p63 evolved earlier to p53 in vertebrates and all three proteins share similar sequence, architecture, and function. Since its discovery in 1997, p73 has been intensively studied due to its high structural similarity to p53 and owning to the possibility to compensate for p53 loss in tumors [54]. p73 has higher than p63 percentage of homology in the DNA binding domain to p53 and forms open tetramers in a manner similar to p53, while p63 forms two closed dimers (**Figure 2 a (lower panel), b**) [55,56]. Such similarity to p53 allows to make an assumption that p73 might recognize and activate many of p53 target genes and that similar pharmacological approaches can be employed to activate p73 protein for improved cancer therapy, which will be discussed in more detail below.

p73 structure

All p53 protein family members are expressed in several isoforms that have distinct functions. The two major p73 isoforms dictating the cell fate upon stress activation and response to chemotherapy are TA isoforms and ΔN isoforms. p73 has two promoters, P1 in the 5′ untranslated region upstream

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of the noncoding exon 1, and P2 within the 23 kb spanning intron 3 triggering the synthesis of two distinct isoforms (reviewed in [57]). TA isoforms are transcriptionally active and act as tumor suppressors and ΔN isoforms, which lack the N-terminus, arise in cells through the alternative promoter usage of P2 and through the alternative splicing. Importantly, when the ratio between the isoforms is altered due to e.g. the methylation of CpG islands in promoter 1, ΔN isoforms accumulate and can interact with and inhibit TA isoforms and p53 [58]. In addition to inhibiting p53 and p73, ΔNp73 has other oncogenic functions like binding to HIF1a and promoting its stability and tumor metastasis [59], driving chemoresistance by regulating the expression of the multi-drug resistance genes ABCB1 and 5 [60], interacting with TGFβ signalling by inducing its target genes PAI-1 and Col1a1[61], inhibiting PTEN tumor suppressor [62] [63]. Additionally, the alternative splicing at the C-terminus generates the C-terminal isomeric forms of p63 and p73 which are expressed both in normal and cancer cells. The longest isoforms TAp63α and TAp73α, contain a highly conserved sterile motif (SAM) (**Figure 2a** (lower panel)), which is a protein-protein interaction module and the p73 SAM domain can bind to anionic and zwitterionic lipid membranes (reviewed in [41]. In total, there are 35 isoforms of p73 which adds complexity while studying p73 biology [64].

Due to high structural homology in DBD (**Figure 2b**), p53 and p73 can transactivate many of the same target genes such as *PUMA*, *CDKN1A*, or *BAX* and maintain the tumor suppressor function by guarding the genomic stability and promoting cell cycle arrest, replicative senescence or apoptosis [65,66]. Reports also point to the involvement of p73 in regulating metabolism due to the accumulation of reactive oxygen species [67].

p73 activity is regulated by a plethora of post-translational modifications like ubiquitination, phosphorylation, acetylation, or sumoylation elicited by stress conditions such as an oncogenic insult or IR-mediated DNA damage [68]. Alike p53, p73 transcription function is inhibited by binding to MDM2 [69] and MDMX [70] through TA domain (**Figure 3**) and the affinities of MDM2 and MDMX to p73 are of the same order as to p53, K_d (μM)=1.4 and K_d (μM)=0.22, respectively [71]. This further highlights a high structural similarity between the domains of p53 and p73 which was confirmed for the TAD domain in a detailed study using molecular dynamics simulations. These studies clearly showed only transient structural fluctuations of the p53 α-helix when in proximity to the MDM2 binding pocket which is stabilized quickly and lasts through the simulation [72]. On the contrary, p63 N-terminus takes a relatively long time to fold into an α-helix which unfolds frequently to a disordered state, whereas p73 α-helix sets like p53 and once is formed, it remains in a set state.

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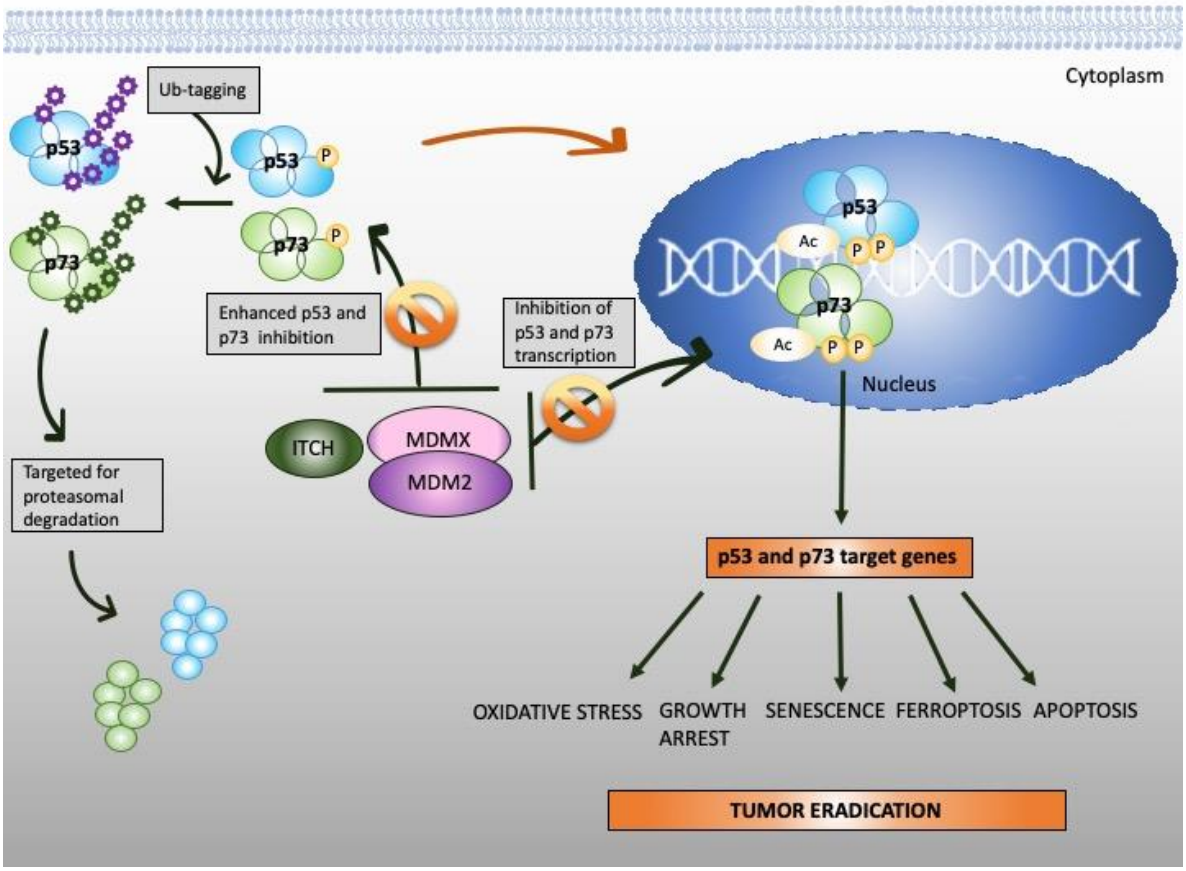

Figure 3. Reinstatement of p53 and TAp73 to treat cancer. Both p53 and TAp73 are rendered inactive in tumor cells through enhanced ubiquitination by MDM2/MDMX and MDM2/MDMX/ITCH axis, respectively. In addition to enhanced protein degradation, the transcriptional activity of p53 and TAp73 is inhibited through binding to MDM2 and MDMX. Targeting protein-protein interactions with small molecules or peptidomimetics (orange, crossed circles) stabilizes p53 and TAp73 and restores their transcription function (orange arrow). This, in turn, promotes tumor eradication through multiple mechanisms, as depicted in the scheme.

iASPP protein, the inhibitor of the apoptosis-stimulating protein of p53 also binds to p73 and inhibits p73-mediated transactivation of apoptotic proteins PUMA and Bax [73]. Of note, the iASPP-p73 complex can be targeted by a p53-derived 37-aa peptide which leads to the induction of p73-dependent apoptosis [74].

p73, as p53, has both transcription dependent and independent functions. Transcription activity of the longest form of p73, TAp73α, similarly to p53, is induced by acetylation by p300 and CREB-binding protein (CBP) acetyltransferases [75]. Yet, p73 transcriptional activity and cell death induction is significantly enhanced by YAP (YES-associated protein) through p300/CBP. Interestingly, YAP stability is enhanced by DNA damage through c-Abl kinase-mediated phosphorylation leading to the reinforced TAp73-mediated apoptosis. Besides, p73 is directly phosphorylated by c-Abl at Tyr99 which promotes its transcriptional activity and highlights the role of TAp73 in maintaining genomic stability [76]. In addition to promoting p73 transcription activity, YAP also outcompetes MDM2 and ITCH E3-ligase, discussed below, from the binding to TAp73 promoting TAp73 protein stability [77].

Similarly to p53, the stability of p73 is mediated by E3 ubiquitin ligase. The major E3 ubiquitin ligase of p73 is HECT ligase ITCH [78]. Next, MDM2 and MDMX both bind to the N-terminal region of p73 and inhibit its transcriptional activity [79,80]. Recent studies indicate that MDM2 promotes p73 proteolytic disassembly through interacting with ITCH [81][82] and that at high levels, MDM2

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polyubiquitinates p73 and plays a role in p73-driven apoptosis [82]. In addition to the ubiquitin-dependent mechanism, both, p53 and p73 can be degraded by 20S proteasome in a ubiquitin independent manner which is inhibited by NQO1 (NADPH quinone oxidoreductase) [83].

p73 has also cytoplasmic, transcription independent functions and after DNA damage induces apoptosis through the noncanonical binding to anti-apoptotic Bcl-XL [84].

p73 tumor suppressor function

After its discovery, the function of p73 in cancer was largely unexplored. Early studies demonstrated that the knockout of *Tp*53 leads to tumor development in mice [85]. The mice heterozygous for *Tp*73 ($p73^{+/-}$) are tumor prone [86], and the studies from the Tak Mak's Lab demonstrated unequivocally that the knockout of TAp73 ($TAp73^{-/-}$) leads to tumor development and infertility *in vivo* [87]. Around 70% of the mice cohort develops lung cancer, and the rest shows premature aging, which is attributed to the de-regulated metabolism. Infertility occurs due to genomic instability. Aberrancy in the DNA repair system in $TAp73^{-/-}$ mice might affect the quality of oocytes in a manner similar to the one occurring during healthy aging and thus, may explain the observed phenotype. This study demonstrated for the first time that TAp73 is a powerful tumor suppressor involved in DNA repair. Next, Elsa Flore's Lab determined that acute genetic depletion of ΔN isoforms of p73 and p63 shrinks tumors developed in the *Tp*53-null background *in vivo* [88]. The mechanism is through the induction of apoptosis. Thus, the deletion of ΔNp73 compensates for p53 tumor suppression and this occurs through upregulation of the TAp73 isoform. Of note, the depletion of $MDM2^{-/-}$ in $Tp53^{-/-}$ null tumors leads to the upregulation of p73, tumor cells' apoptosis and tumour regression *via* activated p73 [89]. Thus, the above-mentioned studies and other [90] using genetically modified models, comprise a large body of evidence that demonstrates that the deregulated p73 tumour suppressor contributes to cancer development and progression and that the increased TAp73 levels compensate for p53 loss in tumor suppression.

Pharmacological targeting of TAp73

Unlike *TP*53, *TP*73 gene is infrequently mutated in cancers [48,91], however, oncogenic ΔNp73 was found to be upregulated in several cancers including gastric and esophageal cancers, thyroid cancer, head and neck squamus cell carcinomas, the cancers of the lung, colon, breast or ovary and is linked to poor prognosis and treatment resistance [92,93].

Whether pharmacological activation of p73 can compensate for p53 loss has been for a long time controversial. Apart from IR-induced DNA damage, only a few molecules have been described to directly or indirectly activate TAp73 in cancers. As mentioned above the epigenetic modification alter the ratio between TA/ΔN isoforms and mutant p53 oligomerizes with TAp73, thus current therapeutic efforts aim at targeting protein-protein interactions to re-activate p73 in tumors.

The very first study using siRNA-mediated inhibition of ITCH demonstrated that cells lacking p53 are more sensitive to ITCH silencing after treatment of chemotherapeutics and undergo rapid apoptosis due to p73 activation [94].

p53 and p73 are subject to similar regulation by MDM2 protein, and researchers showed, that a higher dose of Nutlin-3 efficiently induces TAp73 and apoptosis in cancer cells [95]. Small molecule RETRA, on the other hand, targets mutp53/p73 complex and specifically suppresses the growth of mutant p53-bearing tumor cells *in vitro* and in mouse xenografts [96].

p73 and c-Abl kinase were described to be the components of a mismatch-repair-dependent apoptosis pathway which significantly contributes to cisplatin-induced cytotoxicity in cells harboring wild-type p53 [97]. Interestingly, a study published in the Journal of Clinical Investigation showed that ΔNp63

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mediates p73-dependent sensitivity in triple-negative breast cancer [98]. Next, the study demonstrated that ΔNp63 promoted the survival of breast cancer cells by binding to TAp73 and inhibiting its proapoptotic activity. In addition, breast cancer cells expressing ΔNp63alpha and TAp73 exhibited cisplatin sensitivity that was uniquely dependent on TAp73. In response to treatment with cisplatin, TAp73 underwent c-Abl-dependent phosphorylation, which promoted dissociation of TAp73 from the complex with ΔNp63 and this triggered TAp73-dependent transcription of proapoptotic Bcl-2 family members and apoptosis. This supports the key role of TAp73 in eliminating cancer cells in response to DNA damage activated by cisplatin.

Additionally, the recent study showed that hypermethylation of P1 of p73 correlates with the decrease of TAp73 and shorter overall survival of bladder cancer patients. A DNA demethylating agent, decitabine decreased the methylation of CpGs in P1 of p73 and increased the sensitivity to cisplatin in cell culture conditions [99]. The study from Christian Gaiddon Lab showed that HDAC significantly induces the mRNA and protein levels of TAp73 and p53 on the protein level in gastric cancer cell lines after cisplatin treatment. This leads to the efficient induction of the proapoptotic gene *PMAIP*1 (*NOXA*) and *BIK* [100].

In addition to the extended studies on cisplatin, TAp73 was also described to sensitize p53-null colon cancer cells (HCT 116 $p53^{-/-}$) to withaferin A (WA), a plant-derived proteasomal inhibitor. WA stabilizes and activates TAp73 through the JNK-NQO1 axis and reactive oxygen species-mediated response. In more detail, the study showed that WA induces TAp73 phosphorylation by JNK kinase, releases p73 from MDM2, stabilizes p73 on the protein level, and induces TAp73-dependent apoptosis in p53-null cells. This study further supports the notion that TAp73 efficiently compensates for p53 loss in tumor suppression [101].

A study with bortezomib (Velcade®), a known proteasomal inhibitor approved by the FDA to treat R/R multiple myeloma and as a frontline treatment, was published in 2017. Here, researchers used a pair of isogenic HCT 116 human colon cancer cell lines differing only in p53 status, and showed that TAp73 compensates for p53-loss and induces apoptosis after treatment with bortezomib [102].

Thus, based on the successful reports highlighted above, showing that TAp73 can efficiently compensate for p53 loss, the new strategy aiming at the targeted restoration of TAp73 for improved cancer therapy is therapeutically feasible, and TAp73 is a promising therapeutic target in cancers.

3. Targeting p53 protein family for improved cancer therapy using repurposed drugs

The burdens of current cancer care are; difficult to overcome systemic toxicity of new therapies and the financial burden of innovative interventions and their associated interventions [103]. In 2013 the experts in chronic myeloid leukemia made a strong point about the rocketing prices for cancer drugs that often don't bring the benefit to cancer patients [104]. The universal approach of pricing a new drug appears to be based on the cost of the standard care drug for the given indication plus 10-20%.

The estimated cost of bringing new cancer treatment to the market was assessed to be ~$1 billion [105] and the recent analysis by Vinayak Prasad indicates it is $ 757 mln [106]. At present, many new drugs deliver marginal benefits to the patients at a tremendous cost. For example, bevacizumab mentioned above, in colon cancer costs $ 570 000 per QALY (quality-adjusted life-year) [107] and in the case of immunotherapy, the cost is up to $ 800 000 per QALY. The high costs of cancer drugs constitute a serious financial burden and distress to patients due to high out-of-pocket (OOP) expenses [103].

Taken the long-term toxicity of current cancer care which leads to a higher incidence of primary cancers like sarcoma or leukemia later in life, an aspect severely affecting childhood cancer survivors

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[108,109], there is an urgent need for the establishment of less toxic and far more affordable treatments.

One way to address the toxicity and the financial burden of cancer therapies is to apply the drug repurposing approach. Drug repositioning is based on using existing drugs developed for other indications to treat cancer [110]. A successful example of such approach is all-trans retinoic acid, ATRA, first approved to treat acne and next successfully repurposed to cure acute promyelocytic leukemia [111].

When reconstituted in established tumors, p53 and TAp73 trigger rapid tumor regression *in vivo* [88,112]. Importantly, despite earlier controversies regarding tumor suppressor function of p73, studies described above show that TAp73 efficiently compensates for p53 loss in the induction of apoptosis in cancer cells upon IR or treatment with cisplatin or proteasomal inhibitors. Taken the above and the fact that p53 and p73 bear high structural homology, both members of the p53 family, has become very promising, druggable targets for improved cancer therapy.

Relating to p73 restoration for improved cancer therapy and drug repurposing approach, earlier studies from Gerry Melino's Lab, identified inhibitors of ITCH E3 ubiquitin ligase among anti-depressant drugs [113]. The compounds show anti-cancer activity against lung cancer [114] and might restore TAp73 function in cancer cells and induce apoptosis. However, further studies are needed to evaluate the efficacy of TAp73 re-activation by anti-depressant drugs in tumors.

Protoporphyrin IX (PpIX) is a natural analog of heme, synthesized in the first step from succinyl-CoA and glycine which form aminolevulinic acid (ALA), a heme precursor. This step of heme synthesis is catalyzed by ALA synthase (EC 2.3. 1.37).

The ferrochelatase (FECH, EC 4.99.1.1) is an enzyme required for the incorporation of Fe^{2+} into protoporphyrin IX ring (heme devoid of Fe^{2+}), the terminal step in the heme synthesis pathway. The mutations in the human *FECH* gene (located at chromosome 18q21.3) trigger protoporphyrin accumulation in the skin, erythrocytes, and liver, resulting in light sensitivity. Cutaneous photosensitivity is due to the photosensitizing properties of porphyrins. Porphyrins absorb light at the far UV region, (Soret band) 400-410 nm and to a lesser extent in the long visible Q bands, 580-650 nm, resulting in the generation of the excited electronic states [115]. The reduced activity of the FECH enzyme causes a rare syndrome called erythropoietic porphyria (EPP), an autosomal semi-dominantly inherited disease, which is manifested by the accumulation of protoporphyrin in the erythrocytes, and at a later stage in plasma, from where it is absorbed by hepatocytes and excreted in bile. EPP is the most common erythropoietic porphyria and the symptoms vary depending on the type of mutation in the *FECH* gene and the consequent degree of enzyme inhibition. The symptoms are mostly photocutaneous in the form of non-blistering lesions that develop after sun exposure, however, 5% of patients progress rapidly to liver failure. This is attributed to the inheritance of two heterozygous mutations of *FECH* which ablates the enzyme's activity and thus, leads to the massive accumulation of protoporphyrins in the liver and consequent hepatic damage [116] [117].

EPP can also be acquired, which is linked to the aberrancies in chromosome 18. The recent study demonstrated that the EPP disease was associated with a hematological disease, largely with myelodysplastic syndrome (MDS) with 18q deletion leading to the loss of one of *FECH* allele [118]. Surprisingly, such patients present acute symptoms with immediate painful cutaneous photosensitivity, blistering, and hepatic insufficiency. The severity of the symptoms might be explained either by the co-existing mutations in the remaining allele and further drop in FECH activity or by the aberrancies in heme synthesis pathway and iron metabolism linked to MDS further inhibiting FECH [119]. Of note, other studies demonstrated the reduction of ferrochelatase activity in the malignant tissue by several factors when compared with that in the liver. Thus, FECH inactivation

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explains the observed enhanced accumulation of porphyrins and enhanced autofluorescence of tumors [119–121].

The FECH, and ALA synthase, are both negatively regulated by high concentrations of heme and hemin (Panhematin®), an analog heme possessing oxidized Fe^{3+} and coordinating chloride ligand. Panhematin®, which is applied in treating the acute attacks in porphyria patients as it inhibits heme synthesis, was repurposed to target lung cancer cells through the degradation of heme-binding protein BACH1 [122]. The activity of FECH is also inhibited by divalent cations Mn^{2+} (K_i of 15 microM), Cd^{2+}, Hg^{2+}, arsenite, and by Pb^{2+} [123,124].

Due to the altered activity of FECH in the diseased tissue promoting the accumulation of porphyrins after exogenous administration of ALA, ALA-PpIX is now applied in clinics to treat actinic keratosis in combination with light [125]. Briefly, the administration of γ-ALA salt promotes enhanced synthesis of PpIX in the diseased cells and due to its spectral properties, PpIX becomes excited by light matching the maximum of its absorption. Excitation of PpIX promotes Type 1 and/or Type 2 photoreaction, depending on tissue oxygenation, leading to the generation of reactive oxygen species (ROS) O_2*, H_2O_2 and HO*, or singlet oxygen ($^1O_2$). ROS causes damage to the cells and cells' eradication [126][127]. This procedure is called photodynamic therapy.

Several studies described effective repurposing of PpIX to target cancers. Bednarz et al. showed that enhancing the levels of PpIX in cancer cells by exogenous protoporphyrin IX (exo-PpIX) triggers efficient apoptotic cell death without light activation in HeLa cancer cells [128]. Exo-PpIX also induced apoptosis in murine sarcoma cells. The mechanism of cell death described by authors converges on the decrease of the mitochondrial membrane potential and the release and translocation of mitochondrial AIF factor to the nucleus [129]. Next, exo-PpIX was demonstrated to elevate the levels of p53 and its pro-apoptotic targets in human colon cancer cells HCT 116 leading to the induction of cell death [130]. Zawacka-Pankau et al. showed that p53 activation is due to the disruption of MDM2-p53 complex. The mechanism of complex inhibition is the direct association of PpIX with N-terminus of p53 as depicted using fluorescence correlation spectroscopy. The direct binding of PpIX to N-terminus of p53 was confirmed by my group, in the follow-up studies by gel filtration, anisotropy measurements, and fluorescent band shift assay [131].

The exact mechanism how PpIX interacts with p53 still needs to be elucidated. It is presumed that PpIX, by binding to p53, shifts the conformation of p53 α-helix containing the MDM2 binding residues and renders them unavailable for the interaction with MDM2. Detailed studies are needed to pin down which residues are responsible for the binding of PpIX to p53.

Importantly, the extended studies from my group, further demonstrated that exo-PpIX is an effective dual inhibitor of MDM2-p53 and MDMX-p53 interactions as shown in yeast-based reporter assay, fluorescence two-hybrid assay and immunoprecipitation [132]. PpIX induced apoptosis in leukemia cells without affecting normal cells. PpIX is the only compound reported to date to serve as a dual inhibitor of the p53/MDM2/MDMX interactions which binds to p53.

In addition to p53, the study using a pair of isogenic human colon cancer cell lines HCT 116, differing only in p53 status showed that cells lacking p53 are also sensitive to PpIX without light activation, though at different kinetics. It has been reasoned that in p53-null cells TAp73 might compensate for p53 loss and induce apoptosis. *In vitro* binding studies revealed the binding of PpIX to the p73 N terminal domain [131]. Next, my group showed that PpIX stabilizes TAp73 on the protein level in p53-null cancer cells, induces TAp73-dependent transcription as demonstrated in yeast-based reporter assay and activates apoptotic p73 target genes NOXA, PUMA in cancer cells, which was p73-dependent. Also, PpIX inhibited tumor growth of p53-null subcutaneous xenografts through activation of TAp73 [133] (**Figure 4**).

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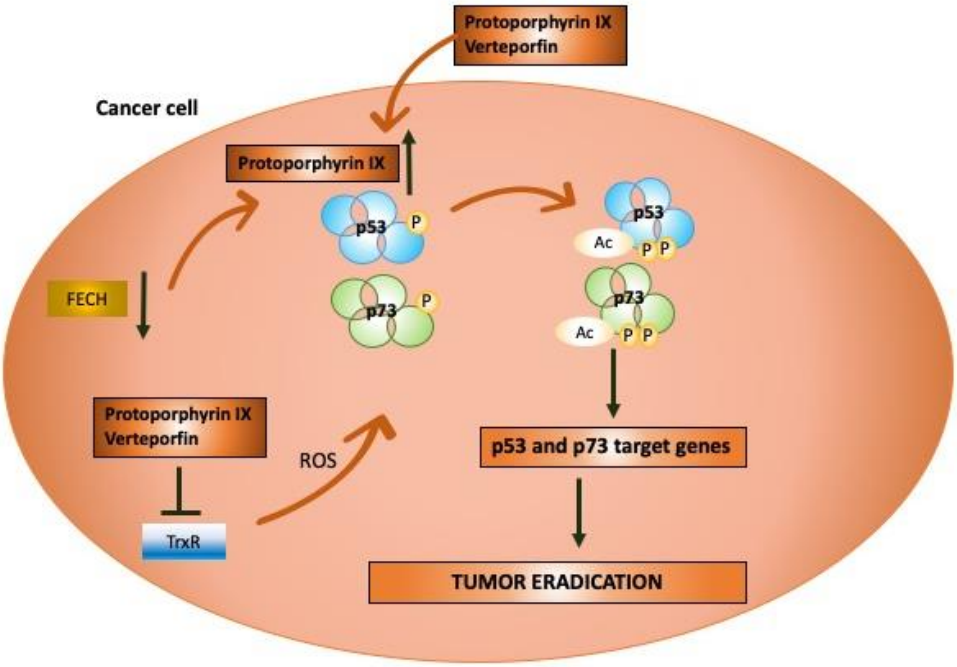

Figure 4. Repurposing porphyrins for improved cancer therapy. The low activity of ferrochelatase in cancer cells leads to elevated levels of PpIX. High levels of PpIX sensitizes malignant cells to exo-PpIX and Verteporfin which bind to p53 and TAp73, stabilize the proteins, and induce p53- and TAp73-dependent transcriptional activity. Inhibition of TrxR (thioredoxin reductase) by PpIX and Verteporfin generates ROS and amplifies p53-dependent and TAp73-dependent apoptosis.

The analog of protoporphyrin IX, benzoporphyrin derivative (BPD, verteporfin), known under the commercial name Visudyne®, is approved by the FDA to treat age-related macular degeneration in combination with light. The compound is listed in the Re_DO database [4] describing off-patent compounds that are promising candidates for drug repositioning in oncology.

Recent studies demonstrated that repurposed PpIX and Visudyne®, stabilize TAp73 on the protein level and induces apoptosis in pancreatic cancer cells harboring mutant *TP*53 gene without affecting non-transformed cells. Besides, the same study showed that both PpIX and Verteporfin induce ROS and are efficient inhibitors of thioredoxin reductase, a component of a thioredoxin-thioredoxin system responsible for forming reduced disulfide bonds in cells, *in vitro* and in pancreatic cancer cells [134]. The mechanism of TAp73 restoration in mutp53 pancreatic cancer cells, as in p53-null cell might be due to inhibition of MDM2-TAp73 and MDMX-TAp73 complex, however, this has not been yet unequivocally shown (Figure 4). Interestingly, BPD was also described to inhibit YAP-TEAD interactions and to decrease liver overgrowth in mice [135].

Yet, further studies are needed to fully elucidate the potential of PpIX and Visudyne® for improved therapy in pancreatic cancer.

A recent study repurposed the FDA-approved anti-malarial drug, amodiaquine, and showed that it stabilizes p53 through inhibition of ribosome biogenesis [136]. Amodiaquine (AQ) was demonstrated to inhibit rRNA transcription, a rate-limiting step for ribosome biogenesis, and to trigger the degradation of the catalytic subunit of RNA polymerase I (Pol I) in the absence of DNA damage. AQ stabilized p53 at low doses and the mechanism was through inhibition of ubiquitin ligase activity MDM2 due to the interaction with the RPL5/RPL11/5S rRNA complex.

The last promising example of drug repurposing is niclosamide. It is an oral salicylanilide derivative approved by the FDA to treat the intestinal tapeworm infections. The drug was shown to overcome the p53 deficiency in cancer cells and manifested anti-cancer activity, specifically in mutant p53 tumors. Niclosamide is a mitochondria uncoupler and stabilizes p53 and induces p53 apoptotic response. Interestingly, the cells lacking p53 were more sensitive to the drug. Further studies showed that the mechanism of apoptosis induction in p53-null cells was mediated by arachidonic acid and that wtp53 decreased its levels in cancer cells, rendering them insensitive to the drug [137]. It is not clear yet if TAp73 contributes to the observed robust induction of apoptosis in cancer cells lacking functional *TP*53, and further studies are needed to address this point.

4. Future perspectives/Conclusions

Despite the documented success of repositioning of all-trans retinoic acid for curing APL, many promising studies on drug repurposing in oncology are still at the early preclinical phase. An exception is the trials with repurposed aspirin, an anti-inflammatory compound, and metformin, a common anti-diabetic drug or sildenafil (Viagra®). In clinical studies, the repurposed drugs are applied alone, in combination with the standard care or with the metronomic chemotherapy. Up to now, there are 179 clinical trials listed for cancer for aspirin and 354 trials for metformin (https://clinicaltrials.gov/ct2/home). Interestingly, metformin was shown in several studies to induce the p53 pathway and p53 is required to induce senescence and apoptosis in breast cancer cells treated with the drug [138].

At present, no clinical studies repurposing ALA-PpIX or Verteporfin without light activation are listed. Taken what we already know about the metabolism of porphyrins in cancer cells and about the mechanism of p53 and p73 reactivation by PpIX and BPD, the compounds are very promising candidates for drug repurposing in oncology. Advanced studies are needed to fully apprehend the mechanism of TAp73 reactivation in tumors with *TP*53 mutations, a great, unmet medical need.

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