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Drugging p53 in cancer: from yeast to human cells

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Abstract

The tumour suppressor p53 is a major transcription factor activated in response to cellular stresses to induce cell cycle arrest, senescence, and apoptosis. *TP53* is the most frequently mutated gene in human cancers. Actually, about half of all human tumours express inactive mutant forms of the p53 tumour suppressor, which often correlates with high resistance to conventional chemotherapy and poor prognosis. Additionally, in tumours retaining a wild-type p53 status, the activity of this protein is suppressed due to the overexpression of two structurally related p53-negative regulators, murine double minute (MDM)2 and MDMX.

The simultaneous inhibition of the interactions of MDM2 and MDMX with p53, resulting in a full p53 reactivation represents a promising anticancer strategy, due to the critical and non-redundant role of these negative modulators. In this work, from the screening of a small library of tryptophanol-derived oxazolopiperidone lactams, using a yeast target-directed approach, a potential dual inhibitor of the p53 interaction with MDM2 and MDMX, the *N*-tosylindole OXAZ-1, was discovered. OXAZ-1 exhibited a p53-dependent *in vitro* antitumour activity against tumours retaining wild-type p53 and distinct levels of MDM2 and MDMX. The promising antitumour activity of OXAZ-1 was reinforced by its ability to trigger a mitochondria-mediated apoptotic cell death and to sensitize tumour cells to the effects of conventional chemotherapeutic drugs. The *N*-tosylindole OXAZ-1 opens the way to the development of a new class of dual MDM2/MDMX inhibitors, with promising applications in anticancer therapy.

An additional outcome of this work was the development of new yeast target-directed screening assays for reactivators of some of the most prevalent human mutant p53 forms, namely R280K, R175H, R273H and Y220C. The pharmacological restoration of the wild-type activity to mutant p53 is an appealing and selective anticancer therapeutic strategy, since besides their high prevalence, expression of p53 mutants is restricted to tumour cells. The developed yeast target-directed screening assays may also represent a relevant tool for the further elucidation of the mutant p53 network, since the biology of p53 mutants remains largely unknown.

Keywords: anticancer agents; p53-MDM2 interaction; p53-MDMX interaction; mutant p53; tumour suppressor proteins; yeast target-directed assays

Resumo

A proteína supressora tumoral p53 representa um dos principais fatores de transcrição ativados em resposta a stresses celulares levando à paragem do ciclo celular, senescência e apoptose. O gene *TP53* é o mais comumente mutado em cancros humanos. De facto, cerca de metade dos cancros humanos expressa uma forma mutada da proteina oncossupressora p53, a qual está frequentemente associada a resistências à quimioterapia e a um mau prognóstico. Para além disso, em tumores que mantêm a forma nativa da p53, a atividade da proteína é suprimida devido à sobrexpressão de dois reguladores negativos estruturalmente relacionados, *murine double minute* (MDM)2 e MDMX.

A inibição simultânea da interação das proteínas MDM2 e MDMX com a p53 nativa representa uma estratégia promissora no tratamento do cancro, tendo em conta as funções distintas e cruciais destes moduladores negativos da p53. Neste trabalho, a partir da análise de uma biblioteca de compostos derivados da família química triptofanol oxazolopiperidona lactamas, e usando o modelo de pesquisa direcionada com células de levedura, foi descoberto um potencial inhibidor dual da interação da p53 com MDM2 e MDMX, o composto *N*-tosilindole OXAZ-1. O OXAZ-1 exibe atividade antitumoral *in vitro* dependente da p53 em células tumorais que possuem a p53 nativa e diferentes níveis de MDM2 e MDMX. A promissora atividade antitumoral do composto OXAZ-1 foi reforçada pela sua capacidade de ativar a via apoptótica mitocondrial e de sensibilizar as células tumorais para os efeitos de quimioterápicos convencionais. O composto *N*-tosilindole OXAZ-1 abre assim caminho para o desenvolvimento de uma nova classe de inibidores duais de MDM2/MDMX, com promissora aplicabilidade em terapias antitumorais.

Para além disso, neste trabalho, foi desenvolvido um novo modelo de levedura para a pesquisa de reativadores de algumas das formas mutadas mais prevalentes da p53 humana, nomeadamente R280K, R175H, R273H e Y220C. A restituição farmacólogica da função nativa da p53 é uma estratégia apelativa e seletiva no tratamento de tumores que expressam mutantes da p53, uma vez que, para além de serem muito prevalentes, a expressão de formas mutadas da p53 é restrita a células tumorais. O modelo de levedura aqui desenvolvido pode também representar uma valiosa estratégia para estudos funcionais das mutantes da p53, uma vez que muitos aspetos biológicos de formas mutadas da p53 continuam amplamente desconhecidos.

Palavras-chave: agentes anticancerígenos; interação p53-MDM2; interação p53-MDMX; p53 mutante; proteínas supressoras tumorais; modelo de levedura para pesquisa direcionada

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Abbreviations

Bax	Bcl-2 associated X protein
Bid	BH3 interacting-domain death agonist
CAK	Cdk-activating kinase
Cdc25C	Cell division cycle 25C
Cdk	Cyclin-dependent kinase
CFU	Colony-forming unit
COX	Cytochrome *c* oxidase
cyt *c*	Cytochrome *c*
DBD	DNA-binding domain
DISC	Death-inducing signaling complex
$DiOC_6(3)$	3,3'-Dihexyloxacarbocyanine Iodide
DMSO	Dimethyl sulfoxide
FADD	Fas-associated protein with death domain
FCCP	Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone
GADD45	Growth arrest and DNA damage protein 45
GOF	Gain-of-function
MDM2	Murine double minute 2
MDMX	Murine double minute x
M-phase	Mitotic phase
MOMP	Mitochondrial outer membrane permeabilization
MMP	Mitochondrial membrane permeabilization
mut	Mutant
OD	Optical density
PUMA	p53 up-regulated modulator of apoptosis
p21	Cdk inhibitor
RE	Responsive element
Rb	Retinoblastoma

ROS	Reactive oxygen species
SRB	Sulforhodamine B
S. cerevisiae	*Saccharomyces cerevisiae*
TAD	Transactivation domain
Y2H	Yeast-two hybrid
wt	Wild-type
$\Delta\psi_m$	Mitochondrial membrane potential

Chapter 1

General introduction

1 General introduction

1.1 Cancer epidemiology and general concepts about cancer

Cancer is the leading cause of death in economically developed countries and the second in developing countries. Despite the improvement in the relative survival rates for many cancer types, cancer prevalence is increasing in economically developed countries as a result of population aging and growth, as well as an increased adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets. According to the World Health Organization, in 2012, there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis). In the same year, in Europe, 3.7 million new cases of cancer were diagnosed and 1.9 million people died with the disease. In Portugal, there were 49.2 thousand new cancer cases, 24.1 thousand cancer deaths and 134.3 thousand people living with the disease (Bray et al., 2012; Ferlay et al., 2013; Soussi and Béroud, 2012). These statistics data may justify why cancer has been the focus of an intense worldwide scientific research.

During development and cellular proliferation there is an intrinsic control system that regulates the balance between cell division and death in response to growth signals. The loss of this balance is the basis of carcinogenesis, a multistage process, involving oncogene activation and tumour suppressor gene inactivation that promotes cell proliferation and/or impairs apoptosis. This process also involves complex interactions between tumour and host tissues, ultimately leading to an aggressive metastatic phenotype [reviewed in (Wang and Sun, 2010)].

A normal cell needs to acquire several characteristics, called hallmarks of cancer, in order to progress to a cancer cell (Figure 1). In 2000, six hallmark capabilities were originally proposed, namely self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Figure 1). However, recent data support that there are two additional hallmarks of cancer, named emerging hallmarks, involved in the pathogenesis of perhaps all cancers. One involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplasic proliferation, and the second allows cancer cells to evade immunological destruction. Additionally, two consequential characteristics of neoplasia facilitate acquisition of both core and emerging hallmarks. Genomic instability and thus mutability endow cancer cells with genetic alterations that drive tumour progression. Inflammation by innate immune cells designed to fight infections and heal wounds can instead result in their inadvertent support of multiple hallmark capabilities, thereby manifesting the now widely appreciated tumour-promoting consequences of

inflammatory responses (Figure 1). Each of these new abilities acquired during cancer development represents the successful breaching of an anticancer defence mechanism hardwired into cells and tissues [reviewed in (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011)].

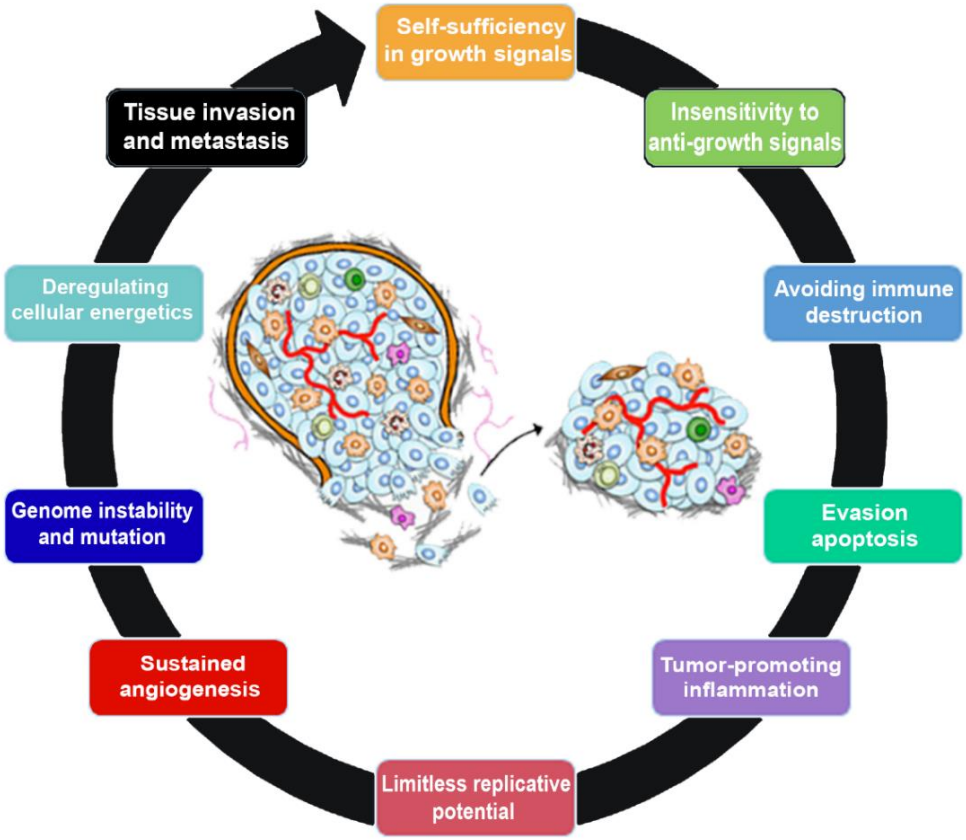

Figure 1. The hallmarks of cancer. This illustration encompasses the ten hallmark capabilities currently proposed. Some of these critical features are: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, genome instability and mutation, sustained angiogenesis, tissue invasion and metastasis [adapted from (Hanahan and Weinberg, 2011)]

1.2 The p53 tumour suppressor protein: A major therapeutic target in cancer

Despite the huge diversity of tumour suppressor genes implicated in carcinogenesis, the *TP53* gene, which encodes the transcription factor p53, has been shown to play a pivotal role protecting cells against cancer development [reviewed in (Suzuki and Matsubara, 2011)].

The *TP53* gene was the first tumour suppressor gene identified. First described in 1979, it was initially reported as a protein interacting with the oncogenic T antigen from SV40 virus (Lane and Crawford, 1979; Linzer and Levine, 1979). Based on these observations, p53 began to be classified as an oncogene, with many studies describing proliferative and transforming activities of the protein. However, this classical point of view has been challenged by the findings that the initially discovered p53 was a mutant (mut) and not the wild-type (wt) form, as was originally considered. Only ten years later, subsequent studies revealed that the wt p53 is capable to suppress the malignant growth of transformed cells as well as tumours, supporting the fact that p53 is a potent tumour suppressor instead of an oncogene [reviewed in (Bourdon, 2007; Freed-Pastor and Prives, 2012)].

The human *TP53* gene, located at position 17p13.1 of the small arm of chromosome 17, has been called the “guardian of the genome”, due to its pivotal role in maintaining genomic stability and tumour prevention [reviewed in (Bourdon, 2007; Freed-Pastor and Prives, 2012)]. Indeed, the p53 tumour suppressor acts as a major regulator in a complex signalling pathway that involves to sense a broad range of cellular stresses such as DNA damage, hypoxia, nutritional deprivation, nucleotide depletion or oncogene activation (Figure 2) [reviewed in (Amaral et al., 2010; Liu et al., 2014; Suzuki and Matsubara, 2011)].

In the absence of cellular stresses, the p53 protein is kept at low steady-levels, through the interaction with murine double minute (MDM) proteins, and exerts little effect on cell fate. However, upon exposure to these countless cellular stress signals, and depending on the tissue-type and the extension of the damage, p53 undergoes post-translational modifications that leads to its stabilization and activation, namely phosphorylation, acetylation and methylation, among others. Once activated, p53 is able to inhibit cell cycle progression, promote senescence, or induce apoptotic cell death, in order to prevent the replication of damaged DNA or the proliferation of genetically altered cells that could lead to tumour formation and development (Figure 2) [reviewed in (Bourdon, 2007; Brosh and Rotter, 2009; Freed-Pastor and Prives, 2012)]. Actually, when the DNA damage is too profound to be successfully repaired by cell cycle arrest, p53 triggers apoptosis. However, when p53 is mutated or absent, damaged cells become resistant to apoptosis and survive (Figure 2) [reviewed in (Amaral et al., 2010)].

The *TP53* gene is inactivated by mutation or deletion in nearly 50% of human cancers. In the remaining cases, p53 retains its wt status but its function is compromised by distinct mechanisms, as the overexpression of its two major negative regulators, the homologs MDM2 and MDMX [reviewed in (Shadfan et al., 2012)].

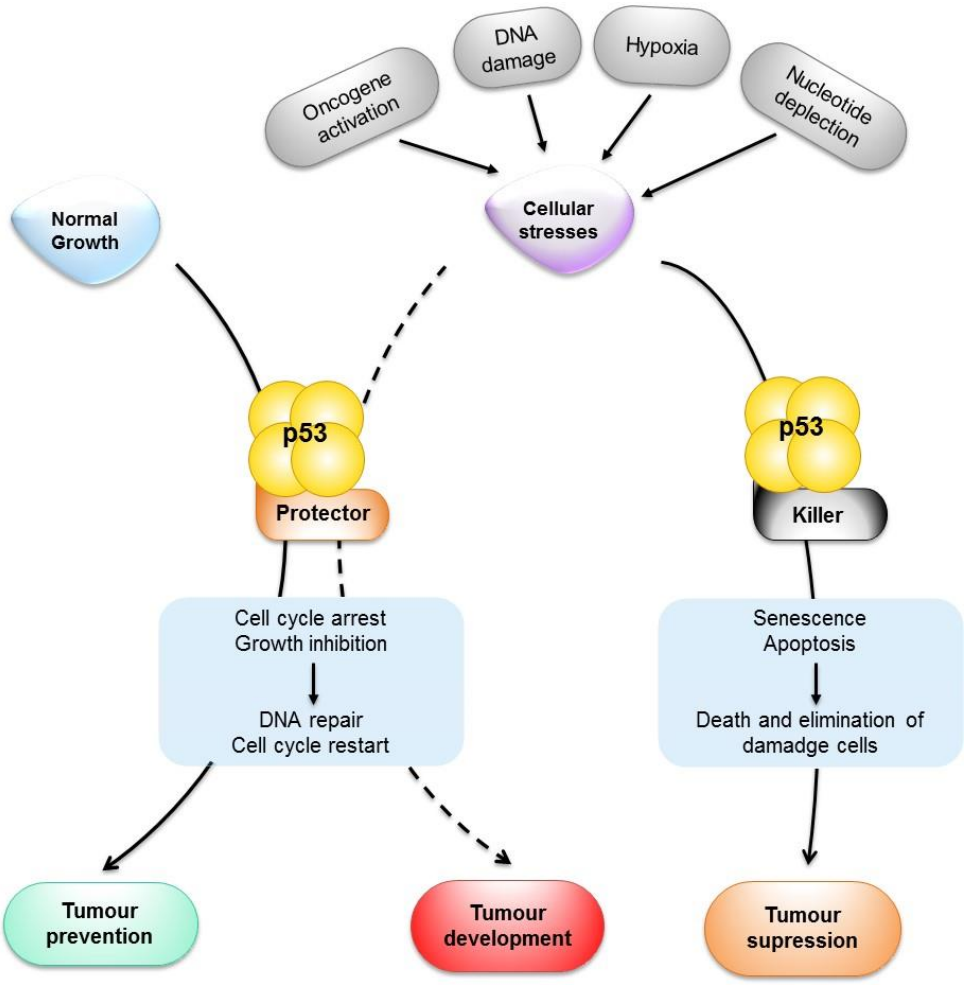

Figure 2. Schematic representation of p53 functions in tumour prevention, suppression and development. When activated by stress signals, p53 can halt the cell cycle and initiate the DNA repair. Under a successful repair, the cell cycle is restarted, and the cellular and genomic stability are re-established. When the damage is too extensive and the repair is not an option, cells induce the killer function of p53 to activate apoptosis or senescence. This activation results in the permanent elimination of damage cells. Both apoptosis and senescence have been linked to tumour suppression, whereas the cell cycle arrest has been linked to tumour prevention. Notably, the protective functions of p53 may contribute to tumour development if not properly regulated (dashed arrow).

Furthermore, the *TP53* gene belongs to a highly conserved gene family containing at least two related genes, *TP63* and *TP73* (encoding p63 and p73, respectively) [reviewed in (Bourdon, 2007; Freed-Pastor and Prives, 2012; Millau et al., 2009)]. Actually, the exogenous expression of p63 or p73 causes growth arrest, apoptosis, and differentiation, which are often due to the transcriptional activation of p53 target genes (Strano et al., 2002). To date, both p63 and p73 have been found rarely mutated in human tumours (Gaiddon et al., 2001; Strano et al., 2002). However, several forms of mut p53 were reported to interact with p63 and p73 through their DNA-binding domain, inhibiting their transcriptional activities (Gaiddon et al., 2001; Strano et al., 2002).

Therefore, the dynamic and multiple functions of p53, highlight the importance in understanding its activity, since the loss of p53 function can lead to more aggressive cancer forms or to chemotherapy resistance making of p53 one of the most appealing therapeutic targets in cancer therapy [reviewed in (Amaral et al., 2010)].

1.3 Structural organization of p53 protein

The 393-amino acid p53 protein has a complex domain structure, which can be schematically divided into three main domains (Figure 3): the *N*-terminal transactivation domain (TAD), the core DNA-binding domain (DBD), the most conserved region of the p53 protein, and the *C*-terminal domain [reviewed in (Millau et al., 2009)]. Each of these domains plays an important role in p53 function.

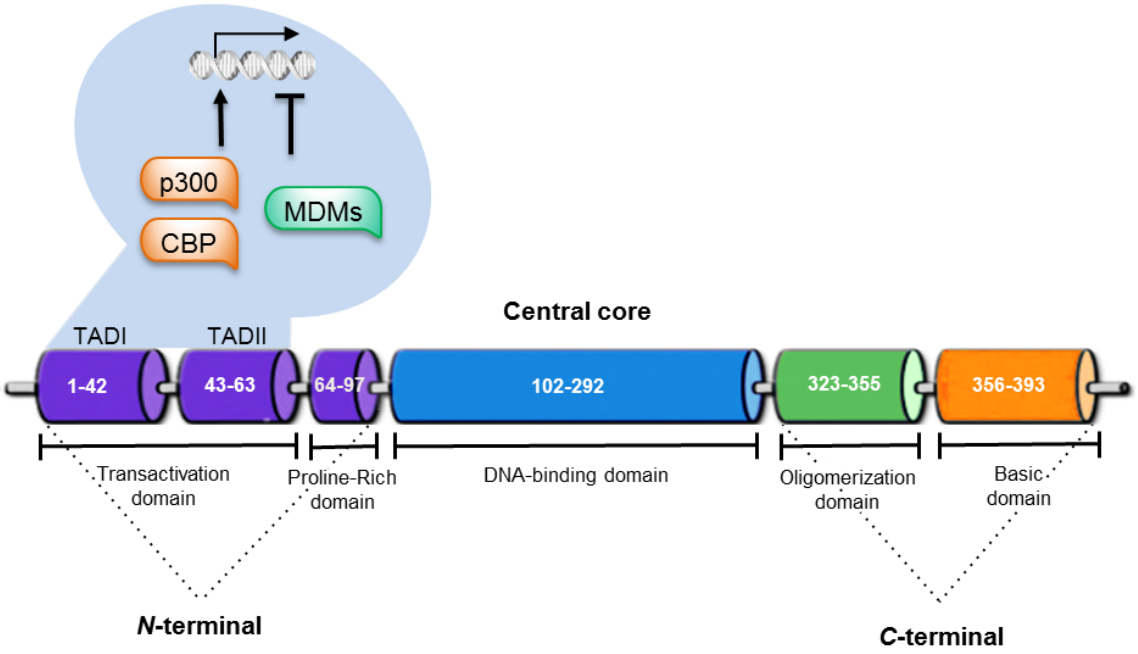

Figure 3. Schematic representation of the domain structure of p53. The human p53 protein comprises several well-defined domains, including an *N*-terminal transactivation domain (TAD), followed by a central core DNA-binding domain, an oligomerization domain and a basic domain at the extreme *C*-terminus. The p53 TAD is able to mediate p53 transcriptional activity and to regulate its stability through interaction with components of the transcription initiation complex, with co-activators, such as p300 and CBP, and with several regulatory proteins, such as MDM2 and MDMX.

The *N*-terminal domain consists of two continuous transactivation subdomains (TADI and TADII) followed by an adjacent proline-rich domain (Figure 3). In fact, it has been shown that the transactivation function of wt p53 is dependent on four critical hydrophobic amino

acids in its *N*-terminus: *Leu22*, *Trp23*, *Trp53*, and *Phe54*. When these four residues are mutated, the transactivation capability of p53 is completely abolished. The p53 TAD is involved in the p53 transcriptional activity and in the regulation of its stability. Both of these capabilities of the TAD are mediated through interaction with components of the transcription initiation complex, with acetyltransferases p300 and CBP (which act as co-activators of p53), and with several regulatory proteins, such as MDM2 and MDMX. Actually, in unstressed cells, p53 would be kept at low levels essentially due to the action of MDMs, which inhibit its transcriptional activity (Figure 3). Upon stress, the *N*-terminus of p53 may suffer modifications that lead to the disruption of its contacts with MDMs. Therefore, p53 can interact with transcriptional co-activators, that leads to modifications, such as acetylation in the *C*-terminus, and allows transcriptional activation and induction of different cellular responses (Figure 3) [reviewed in (Freed-Pastor and Prives, 2012; Joerger and Fersht, 2007)]. Although its precise function has not yet been clearly defined, the proline-rich domain seems to be involved in growth suppression and apoptosis and its deletion results in a complete loss of this function (Sakamuro et al., 1997; Walker and Levine, 1996). Besides that, it is involved in p53 stabilization regulated by MDM2, since p53 becomes more susceptible to degradation if this region is deleted. It has also been reported that this domain comprises a negative auto-regulatory domain reducing the DNA-binding ability (Muller-Tiemann et al., 1998).

The DBD is responsible for the binding to sequence-specific DNA elements, located close to promoters of the p53 target genes. Indeed, this ability of p53 to bind to sequence-specific DNA elements is tightly linked to its pro-apoptotic activity [reviewed in (Ozaki et al., 2013)].

The *C*-terminal domain contains an oligomerization domain, involved in the ability of p53 to form active tetramers, followed by a basic domain, that interacts directly with single strands DNA (Figure 3) [reviewed in (Millau et al., 2009)].The basic domain of p53, acts as a flexible linker region that connects the DBD and the *C*-terminus of p53 (Cho et al., 1994). Besides that, the basic region of the *C*-terminus, which contains acetylation sites that binds DNA non-specifically, acts as a negative regulatory domain, and has been implicated in the induction of cell death [reviewed in (Joerger and Fersht, 2007)].

Interestingly, p63 and p73 share a significant homology with each other and with p53, having the same modular organization, which comprises an *N*-terminal TAD, a DBD and a *C*-terminal oligomerization domain and, consequently, similar functions (Strano et al., 2002).

1.3.1 The p53 role in cell cycle regulation

One of the most important cellular processes regulated by p53 is the cell cycle progression. This process consists on a series of steps at which the cell checks for the accuracy of the process and instructs itself to proceed to the next step. Cell cycle consists in a G1-phase, in which cells increase in size, in a S-phase, where the DNA is duplicated, in a G2-phase, in which cells continue to grow, and in a mitotic phase (M-phase), where chromosome segregation and cells division occurs. The G1 and G2 phases also provide the cell more time to grow and to ensure that the conditions are suitable for entry into the S-phase or mitosis [reviewed in (Nurse, 2000)].

The cell cycle is tightly regulated by stimulators and inhibitors containing intrinsic checkpoint controls, preventing in this way the transmission of damaged genetic material to the daughter cells and the consequent replication of abnormal material. Thus, there are three cell cycle checkpoints that regulate cells progression through each stage: the G1/S, the intra-S and the G2/M checkpoints. In case of DNA damage, the G1 checkpoint will lead to cell cycle arrest, ensuring that the DNA is not replicated during the S-phase. In response to damaged and/or un-replicated DNA the G2 checkpoint leads to cell cycle arrest safeguarding the proper completion of S-phase. The M-checkpoint leads to the arrest of chromosomal segregation in response to misalignment on the Mitotic spindle [reviewed in (Morgan, 1995)].

The cell cycle checkpoints are regulated by cyclin-dependent kinase (Cdk) complexes formed through association of a Cdk and a regulatory protein, a cyclin, that controls and activates the Cdk and whose levels change in a cyclical manner. So, since Cdk levels are usually constant throughout the cell cycle, its regulation and progression depends on variations of the levels of each cyclin (Sheaff et al., 1997). These cyclin/Cdk complexes regulate the phosphorylation of proteins involved in cell cycle progression, leading to DNA replication and mitosis. There are two types of cyclin/Cdks that regulate the transit of mammalian cells from quiescence into S-phase: the cyclin D, which activate Cdk4/6, and the cyclin E, which activates Cdk2 (Figure 4A) [reviewed in (Sherr and Roberts, 1999)].

Depending on the type of the cellular stress, the p53-mediated cell cycle arrest may be considered the primordial response to DNA damage. Actually, in response to a variety of stress conditions, such as chemotherapeutic treatments, p53 can trigger protective, pro-survival responses, such as a temporary cell cycle arrest at the G1-phase, regulation of the G2/M transition, DNA repair and antioxidant protein production to maintain the genome integrity and the viability of cells that sustain limited reparable damage. All this is possible due to the ability of p53 to up-regulate the expression of several cell cycle proteins, such as GADD45 (Growth arrest and DNA damage protein 45), Cdc25C (Cell division cycle 25C)

and p21 (cdk inhibitor also known as CIP1/WAF1) (Figure 4A and B) [(Amundson et al., 1998; El-Deiry, 1998); reviewed in (Nag et al., 2013)].

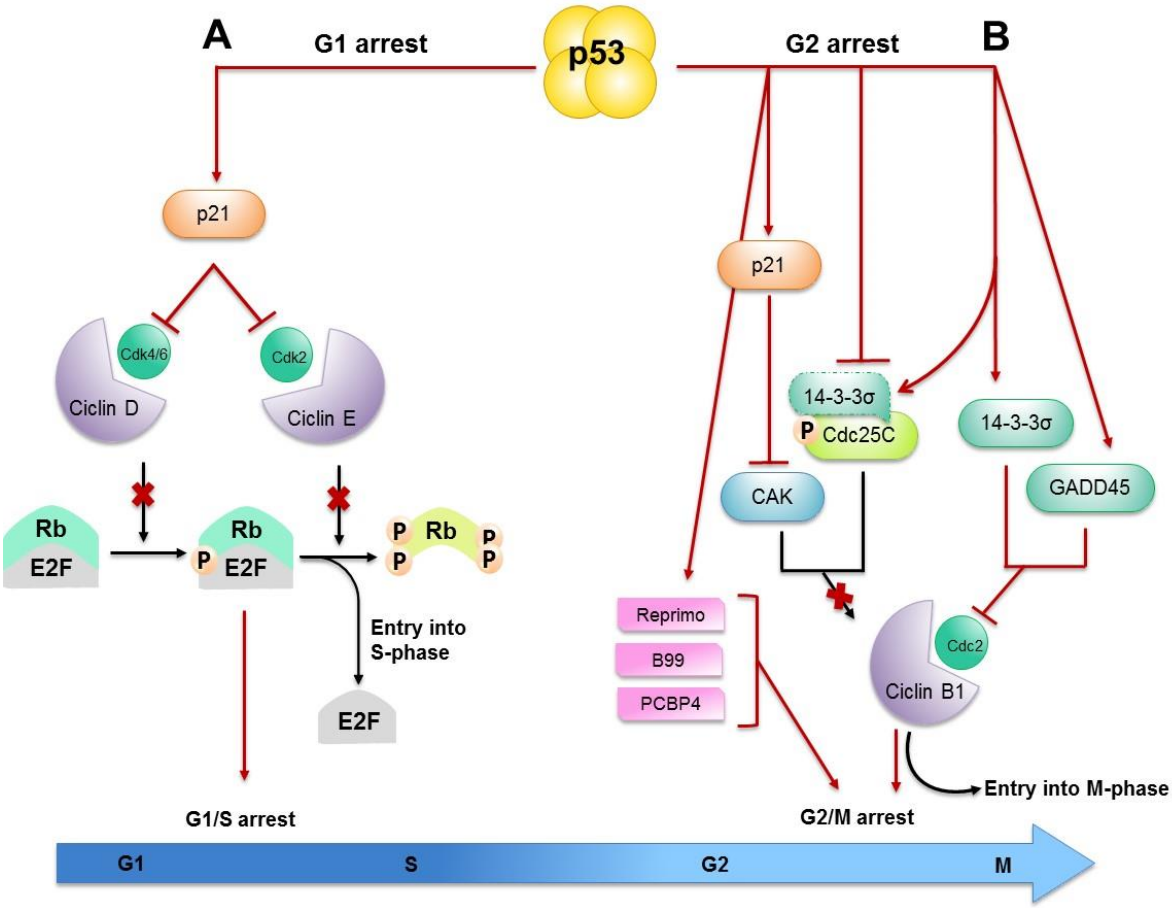

Figure 4. Regulation of cell cycle by p53, which transcriptional activation may lead to growth arrest at both G1 and G2 cell cycle phases. A) Under normal conditions the cyclin/Cdk complexes are functional and Rb family members are found hyperphosphorylated (yellow) leading to the release and activation of the E2F transcriptional factor and, consequently, to G1 progression. Under stress conditions (red arrows) p53 mediates the transcriptional induction of p21, which plays a pivotal role in mediating the arrest at G1-phase. p21 binds to, and inhibits, cyclin/Cdk complexes resulting in the accumulation of hypophosphorylated Rb (green) that interact with and inhibit E2F transcription factors activity. These results in an arrest at the G1-phase. **B)** p53 has also been implicated in the control of the G2/M checkpoint. Transcriptional down-regulation of cyclin B1 by p53 may be involved in p53-mediated G2 arrest, once that cyclin B1/Cdc2 complex is the major regulatory factor required for entry into mitosis. p53 is able to control G2 arrest by inhibition of Cdc25C and CAK, both required for activation of cyclin B1/Cdc2 complex. CAK can be inhibited by p21 and Cdc25C by phosphorylation after activation of the Chk1 and Chk2 kinases, establishing a bind site to 14-3-3σ (green) proteins and leading to inactivation of Cdc2 activity. Besides that, the progression into the M-phase requires Cdc2 which can be inhibited by overexpression of p21, GADD45 or 14.3.3σ, being the expression of these inhibitory proteins regulated by p53 in order to induce growth arrest. p53 has also other targets that do not affect cyclin B1/Cdc2 complex but contribute to G2 arrest, namely Reprimo, B99 and poly(rC) binding protein 4 (PCBP4); P – phosphorylation; All the red arrows and crosses correspond to the p53 effect on cell cycle progression.

During the G1-phase, the expression of cyclin D is stimulated, forming cyclin D/Cdk4/6 complexes that phosphorylate the retinoblastoma (Rb) protein. Under normal conditions, the exit from G1 to S-phase requires the phosphorylation of Rb protein by cyclin/Cdk complexes. This results in the activation of the S-phase-promoting E2F transcription factor (required for entry into the S-phase), which binds to hypophosphorylated Rb protein (Figure 4A) (Amundson et al., 1998; Chellappan et al., 1991). One of the target genes of E2F is cyclin E, which forms a complex with Cdk2 that phosphorylates and consequently inhibits the Rb protein, leading to DNA replication and progression though cell cycle (Koff et al., 1992). Under stress conditions, p53 induces the transcriptional activation of p21 (major effector of G1 arrest), which in turn inactivates the cyclin/Cdk complexes. This enables the activation of Rb proteins and leads to the inhibition of E2F transcription factors (Figure 4A) (El-Deiry, 1998).

The p53 protein is able to regulate the G2/M transition, blocking cell entry into mitosis by inhibition of Cdc2 (Cdk1), which needs to bind to cyclin B1 in order to be functional. Thus, the repression of cyclin B1/Cdc2 complex by p53 leads to an arrest in G2/M transition (Figure 4B) [reviewed in (Suzuki and Matsubara, 2011)]. Additionally, the biochemical pathways involved in the DNA damage-induced G2 arrest involve signalling cascades that converge to inhibit the activation of Cdc2. Therefore, p53 impacts the G2/M checkpoint by transcriptional modulation of the expression of multiple, physically and functionally intertwined targets, including Cdc25C, 14-3-3σ, p21, and GADD45 (Figure 4B) (El-Deiry, 1998; Giono and Manfredi, 2006; Zhan et al., 1998). Mechanistically, p53 leads to G2/M arrest by repressing Cdc25C, a mitosis promoting phosphatase, which dephosphorylates and activates the cyclin B1/Cdc2 complex. In addition, the Cdc25C activity can be inhibited via phosphorylation by checkpoint kinases, Chk1 and Chk2, which, in turn, generate a consensus binding site for 14-3-3σ proteins. Binding of 14-3-3σ protein to Cdc25C results in the nuclear export of Cdc25C, sequestration of the phosphatase in the cytoplasm and, consequent inhibition of Cdc25C (Figure 4B) [reviewed in (Taylor and Stark, 2001)].

Besides the activity of Cdc25C, the Cdk-activating kinase (CAK) phosphorylates specific amino acid residues required for the activation of the cyclin B1/Cdc2 complex [reviewed in (Kishimoto and Okumura, 1997; Sherr and Roberts, 1999)]. Interestingly, some studies suggest that p21, involved in G1 arrest, is also implicated in G2 arrest by disruption of cyclin B1/Cdc2 complex through CAK (Figure 4B) (Smits et al., 2000; Zhan et al., 1999).

The p53 protein has been reported to transcriptionally activate GADD45, which dissociates cyclin B1/Cdc2 complex by binding to Cdc2 [reviewed in (Taylor and Stark, 2001). This prevents the formation of the protein complex necessary for entry into the M-phase, resulting in G2 arrest (Zhan et al., 1999). p53 can also induce the expression of other

proteins involved in G2 arrest, namely Reprimo (a glycosylated, cytoplasmic protein that plays a role in cyclin B1/Cdk1 localization), B99 (a protein that may cause G2 arrest independently of Cdc2) [reviewed in (Taylor and Stark, 2001)] and poly(rC) binding protein 4, a RNA-binding protein (Zhu and Chen, 2000) (Figure 4B).

1.3.2 Apoptosis regulation by p53 protein

Apoptosis is an evolutionary conserved and genetically controlled process of cell death that is essential, not only for development and maintenance of tissue homeostasis, but also for the elimination of unwanted cells during normal development and as a component of multistep carcinogenesis and therapy treatment resistance [reviewed in (Amaral et al., 2010)]. This cellular mechanism is induced by a tightly regulated suicide programme characterized by several hallmarks as cell shrinkage, condensation and fragmentation of nuclear chromatin, DNA fragmentation, mitochondrial swelling and membrane blebbing as well as by loss of mitochondrial membrane integrity. This mechanism of cell death is considered very “clean” occurring without stimulation of an inflammatory response [reviewed in (MacFarlane, 2003; Sharp et al., 2010)].

Alteration of many proteins involved in the apoptotic signalling pathways have been described and it is clear that alterations of upstream regulators of these pathways are the most common alterations found in cancer cells. As an example p53, that can induce cell death in response to a number of different stress stimuli, regulates the expression of a wide variety of genes involved in apoptosis [reviewed in (Vaseva and Moll, 2009)]. The apoptotic function of p53 has been first reported in mouse thymocytes, in response to irradiation (Clarke et al., 1993; Lowe et al., 1993). Since then, the p53-dependent apoptosis has been reported in a wide range of cells in response to many different stress signals [reviewed in (Amaral et al., 2010)].

p53 stimulates a wide network of signals that can act through two major apoptotic pathways (Figure 5): the extrinsic (or death receptor) and the intrinsic (or mitochondrial) [reviewed in (Amaral et al., 2010; MacFarlane, 2003)]. The apoptotic extrinsic pathway is triggered by extracellular signals that result in the binding of ligands to specific trans-membrane death receptors located at the plasma membrane, such as KILLER/DR5 and Fas (also called APO-1). Fas is a member of the tumour necrosis factor receptor (TNFR) superfamily and, in turn, KILLER/DR5 is a member of the TNF-related apoptosis-inducing ligand (TRAIL) family of death receptors [reviewed in (Ashkenazi and Dixit, 1998; Haupt et al., 2003; Sharp et al., 2010)]. The activation of these receptors, after binding to their ligands, leads to their trimerization and consequent clustering of the intracellular death domain, which

subsequently recruits the protein adaptor FADD (Fas-associated protein with death domain) through homotypic death domain interactions. The death effector domain of FADD then recruits procaspase-8 forming the death-inducing signalling complex (DISC) (Yu et al., 2001). The formation of the DISC complex results in caspase-8 activation and consequently in executioner caspases activation (Figure 5A), resulting in DNA fragmentation as an hallmark of apoptosis [reviewed in (Schuler and Green, 2001)]. The p53 overexpression enhances cell surface levels of Fas by promoting its trafficking from Golgi complex. Thus, p53 status may influence the chemosensitivity via Fas signalling [reviewed in (Suzuki and Matsubara, 2011)] and activate KILLER/DR5, which is induced in response to DNA damage and, consequently, promote cell death through caspase-8 activation (Liedtke et al., 2003) (Figure 5A).

On the other hand, the intrinsic apoptotic pathway can be activated in response to a variety of cellular insults or other damaged signals such as radiation. This apoptotic pathway is regulated by members of the Bcl-2 family and involves the disruption of mitochondrial membrane integrity and subsequent release of apoptogenic proteins from the mitochondria into the cytosol (Voortman et al., 2007). The disruption of mitochondrial membrane integrity results in biochemical and structural changes of mitochondria including mitochondrial swelling, changes at the mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial membrane potential ($\Delta\psi_m$), and cytochrome *c* (cyt *c*) release (Figure 5B). Actually, it has been proposed that mitochondrial depolarization defines an early and irreversible stage of apoptosis preceding other manifestations in this process, such as DNA fragmentation and reactive oxygen species (ROS) production. Additionally, the increase of $\Delta\psi_m$ is often observed in human cancer cells (Li et al., 1998; Vrablic et al., 2001; Zamzami et al., 1995).

Therefore, MOMP is essential for the regulation of the apoptotic process and the proteins of the Bcl-2 family are the main regulators of this deadly switch (Renault et al., 2013). The members of the Bcl-2 family include anti-apoptotic, such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as Bax, Bak, Bid, NOXA and PUMA (p53 Up-regulated Modulator of Apoptosis), that can be classified on basis of structural similarity to the BH (Bcl-2 Homology) domains (BH1, BH2, BH3 and BH4) and a transmembrane domain, being the BH3 domain crucial for the pro-apoptotic function [reviewed in (Amaral et al., 2010; Igney and Krammer, 2002; Manfredi, 2003)]. Bax and Bak are the direct pro-apoptotic effectors of MOMP as they can translocate and/or insert into the outer mitochondrial membrane, oligomerize and form pores, leading consequently to cyt *c* release. Despite Bax and Bak are functionally redundant proteins, Bak has a constitutive mitochondrial localization in healthy cells, whereas Bax is mostly cytosolic, awaiting for activation signals that will trigger

its translocation to mitochondria and further activation (Figure 5B) (Renault et al., 2013). Bax activation is favoured by the pro-apoptotic BH3-only proteins, who can act both as direct activators of Bax (tBid, Bim, PUMA), by interacting directly with him, or as derepressors (Bad and Noxa), by interacting with anti-apoptotic members of the Bcl-2 family. The anti-apoptotic family members bind the BH3 domains of pro-apoptotic family proteins, preventing Bak/Bax homo-oligomerization and BH3-only proteins activation. The overexpression of anti-apoptotic members has been reported in most human cancers, favouring survival of neoplastic cells and consequently the resistance to chemotherapeutic agents [(Gallenne et al., 2009; Renault et al., 2013); reviewed in (Vaseva and Moll, 2009)]. Curiously, the binding region for Bcl-xL and Bcl-2 on p53 is located in the DBD, the same region that harbours the vast majority of "*hotspot*" mutations found in human cancers. Indeed, it was shown that such mut p53 proteins are defective in their ability to interact with Bcl-xL and Bcl-2 family of proteins (Mihara et al., 2003).

After several studies, it is clearly established that in response to a stress signal, cytoplasmic p53 rapidly translocates to mitochondria, where it can promote apoptosis in a transcriptional-independent manner, preceding the loss of $\Delta\psi_m$ (Mihara et al., 2003). At the mitochondria, p53 is able to interact, either to inhibit or activate, with several anti- and pro-apoptotic genes of the Bcl-2 family, such as *Bcl-2, BAX, NOXA, PUMA* and *BID* (BH3-only proteins). Indeed, by directly interact with the anti-apoptotic proteins Bcl-xL and Bcl-2, p53 is able to inhibit these proteins inducing cyt *c* release [reviewed in (Amaral et al., 2010; Igney and Krammer, 2002; Manfredi, 2003; Vaseva and Moll, 2009)]. These interplay between pro- and anti-apoptotic members of the Bcl-2 family, after p53 activation, has been found to control the MOMP, and consequently the cyt *c* release from the mitochondria intermembrane space into the cytosol, unleashing the activation of the apoptotic machinery of caspases, chromatin degradation and apoptosis (Figure 5B) (Xiao et al., 2005). Once in the cytosol, cyt *c* participates in the formation of the apoptosome complex together with its adaptor molecule, Apaf-1 (Apoptotic Protease-Activating Factor-1), which activates procaspase-9 and promotes activation and cleavage of executioner caspases (procaspase-3, -6 and -7) (Figure 5B). This event leads to the cleavage of other specific death substrates, cellular and nuclear morphological changes, and ultimately, to cell death (Li et al., 1998). Thus, it is logical to assume that cyt *c* release is the rate-limiting step in initiating the caspase activation cascade and the main consequence of p53-mediated signals leading to apoptosis (Shinoura et al., 2001). Additionally, PUMA may play a pivotal role in determining cell fate (programmed cell death *versus* cell cycle arrest) in response to p53 activation, being an essential mediator for p53-dependent apoptosis *in vivo* (He et al., 2013).

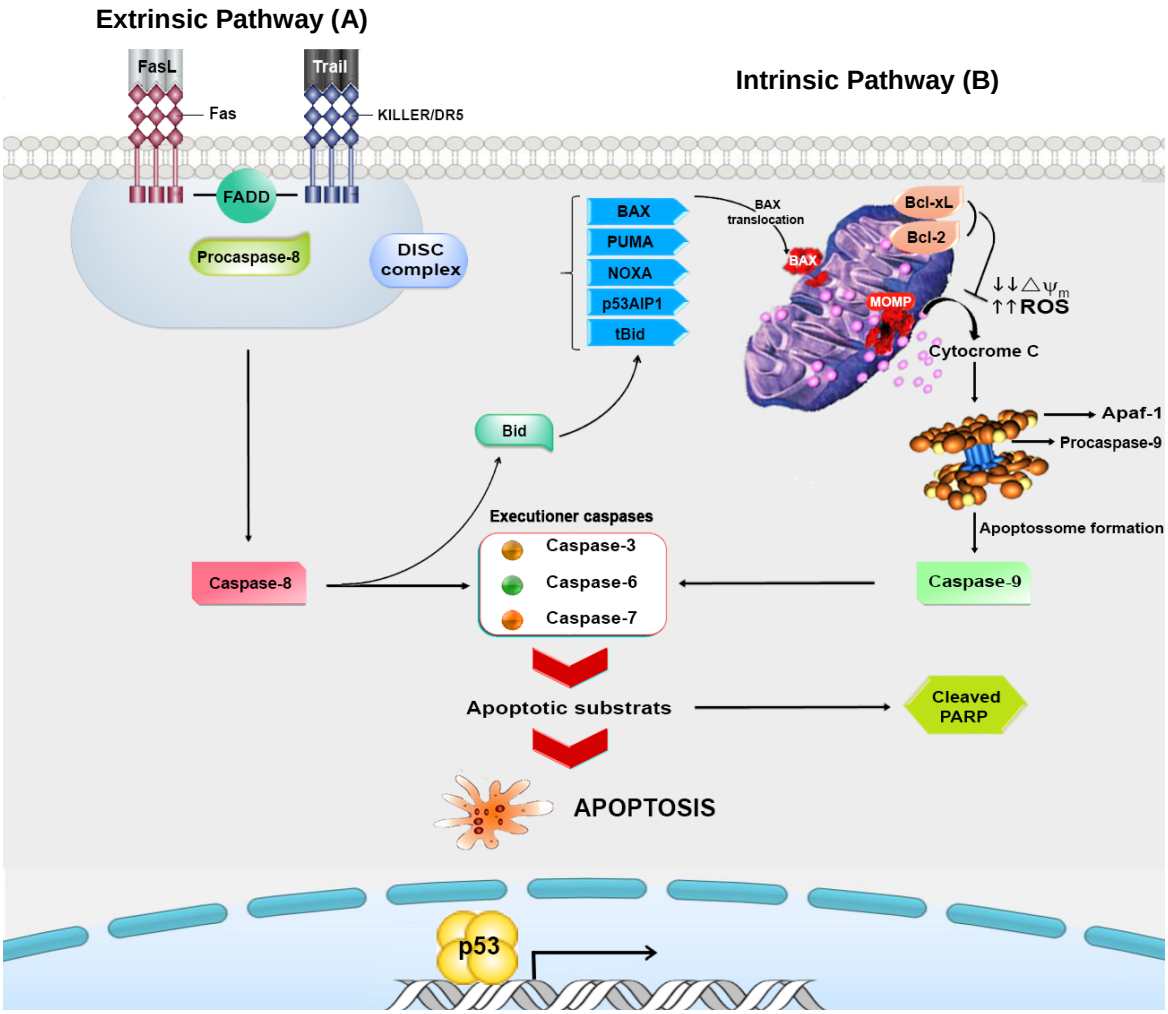

Figure 5. Schematic representation of the regulation of intrinsic and extrinsic apoptotic pathways by p53. The p53 protein is able to induce transcription of proteins involved in both apoptotic pathways. **A)** In the extrinsic pathway, the binding of ligands to specific trans-membrane death receptors (Fas and KILLER), leads to the recruitment of the adaptor molecule, FADD. Then, the procaspase-8 binds to FADD leading to DISC formation and resulting in caspase-8 activation. Activated caspase-8 directly activates executioner caspases (caspase-3, -6, and -7) or cleaves the pro-apoptotic protein Bid. **B)** In the intrinsic/mitochondrial pathway the stress-induced mitochondrial translocation of p53 results in interactions between several anti- and pro-apoptotic members of the Bcl-2 family in order to induce MOMP and cyt *c* release. Indeed, p53 interacts with Bcl-xL/Bcl-2 blocking their inhibitory effect on pro-apoptotic Bax. Besides that it is able to activate genes such as *PUMA* and *NOXA,* which binds to and inhibit the activity of the anti-apoptotic proteins Bcl-2/Bcl-XL, unleashing the translocation of cytosolic Bax to the mitochondria. This leads to mitochondrial permeabilization and activation. The release of cyt *c* from the mitochondria results in the formation of the apoptosome and caspase-9 activation. Then, both caspase-8 and -9 activate executioner caspases, namely caspases-3, -6 and -7, resulting in cell death. Additionally, the MOMP can lead to a loss of the $\Delta\psi_m$ and to increase ROS production [adapted from (Haupt et al., 2003)].

Interestingly, there are evidences that the two apoptotic pathways are not exclusive. Instead, they can be linked and molecules in one pathway can influence the other. In fact, both pathways activate caspases, proteolytic enzymes functionally divided into two groups (initiator and executioner) that carry out apoptotic cell death. Once initiator caspases (such

as caspases-8, -9 and -10) are activated, they cleave and activate (directly or indirectly) executioner caspases (such as caspase-3, -6 and -7) that, in turn, cleave intracellular substrates, such as poly(ADP-ribose)polymerase (PARP) (Figure 5A and B), leading to biochemical and morphological changes that are characteristics of apoptosis. This makes the activation of initiator caspases a key step to cell death [reviewed in (Igney and Krammer, 2002; MacFarlane, 2003)]. One of the major links between the two pathways is provided by the pro-apoptotic Bcl-2 family member Bid. Activation of Bid can promote MOMP and involves cleavage of cytoplasmic Bid by caspase-8 to expose a new *N*-terminal glycine residue that is subsequently myristoylated. Myristoylated Bid translocates to the mitochondria, inserts into the membrane and activates Bax and Bak to initiate mitochondrial events leading to apoptosome formation. The *BID* gene is transcriptionally regulated by p53 in response to γ-irradiation. Therefore, p53 appears to promote the convergence of the intrinsic and extrinsic pathways through Bid regulation (Li et al., 1998; Zha et al., 2000).

Finally, increased mitochondrial production of ROS, has been related to the loss of $\Delta\psi_{m,}$ in cancer cells, but not in normal cells (Gogvadze et al., 2008). While normal levels of ROS can be pro-proliferative, excess of ROS production can damage DNA and proteins, contributing to the development of various diseases (Maddocks and Vousden, 2011).

Interestingly, it was showed that p53 protects cells from oxidation by reducing the production of intracellular ROS (Bensaad et al., 2006). Although ROS are by-products of normal mitochondrial function, high levels of ROS have been associated with p53-induced apoptosis (Figure 5B). The p53 deficiency in cells and mouse tissues results in the elevation of intracellular ROS levels, which in turn leads to the increased DNA oxidation and mutation rates in cells (Budanov et al., 2004; Sablina et al., 2005).

1.4 Endogenous negative regulators of p53: MDM2 and MDMX

Under normal conditions, p53 remains at low levels due to its short half-life allowing normal cell proliferation and viability. However, in response to cellular stresses, p53 becomes stabilized and modulates the transcription of target genes. As referred above, in tumours in which p53 is not mutated, its function is inhibited by overexpression of its two major negative regulators MDM2 and MDMX. Actually, both of them regulate the p53 activity by inhibiting its transcriptional activity (Jones et al., 1995; Parant et al., 2001).

The *MDM2* gene encodes a protein that was first described in the late 1980s as an amplified gene responsible for the spontaneous transformation of an immortalized murine cell line, BALB/c 3T3 (Cahilly-Snyder et al., 1987). Within 5 years, the oncogenic potential of *MDM2*

gene was demonstrated, with several studies showing that MDM2 interacts directly with p53 and inhibits its ability to function as a transcriptional activator [reviewed in (Manfredi, 2010)]. In the middle 90s, a screen for p53-binding proteins led to the identification of a protein sharing a structural homology with MDM2. This protein was first called MDMX and later given the official name of MDM4. The high level of structural similarity makes us thought that these two proteins derived from the duplication of a single ancestral gene [reviewed in (Toledo and Wahl, 2006; Toledo and Wahl, 2007)].

The *MDM2* gene is overexpressed in a wide range of human cancers expressing wt p53 including breast carcinomas, glioblastomas and sarcomas, whereas overexpression of *MDMX* can be detected in melanomas, retinoblastomas and breast carcinomas, being both associated with resistance to treatment, poor prognosis, metastasis and advanced forms of the disease [reviewed in (Nag et al., 2013)].

The full-length transcripts of *MDM2* and *MDMX* genes encode structural related proteins of 491 and 400 amino acids, respectively (Figure 6). Thus, both MDMs bind to a short α-helical stretch on the p53 TAD domain through their *N*-terminal hydrophobic region, which is the best conserved region between the two proteins [reviewed in (Toledo and Wahl, 2007; Wang and Jiang, 2012)]. By blocking the p53 TAD domain and occupying the binding site of co-activators, such as p300 [reviewed in (Hock and Vousden, 2014)], these proteins lead to the inhibition of the p53 transcriptional function (Kussie et al., 1996; Momand et al., 1992). Both MDM2 and MDMX possess a zinc-finger domain together with a central acidic domain, followed by a *C*-terminal RING-finger domain, with this last domain receiving special attention for its multiple functions (Fang et al., 2000). Additionally, only MDM2 possesses a nuclear localization signal (NLS) and a nuclear export signal (NES), responsible for shuttling MDM2 between the nucleus and the *cyt*oplasm (Figure 6). This suggests that MDM2 binds to p53 in the nucleus and transports it to the cytoplasm for ubiquitin-mediated degradation by the proteasome [reviewed in (Freedman et al., 1999)] (Figure 7).

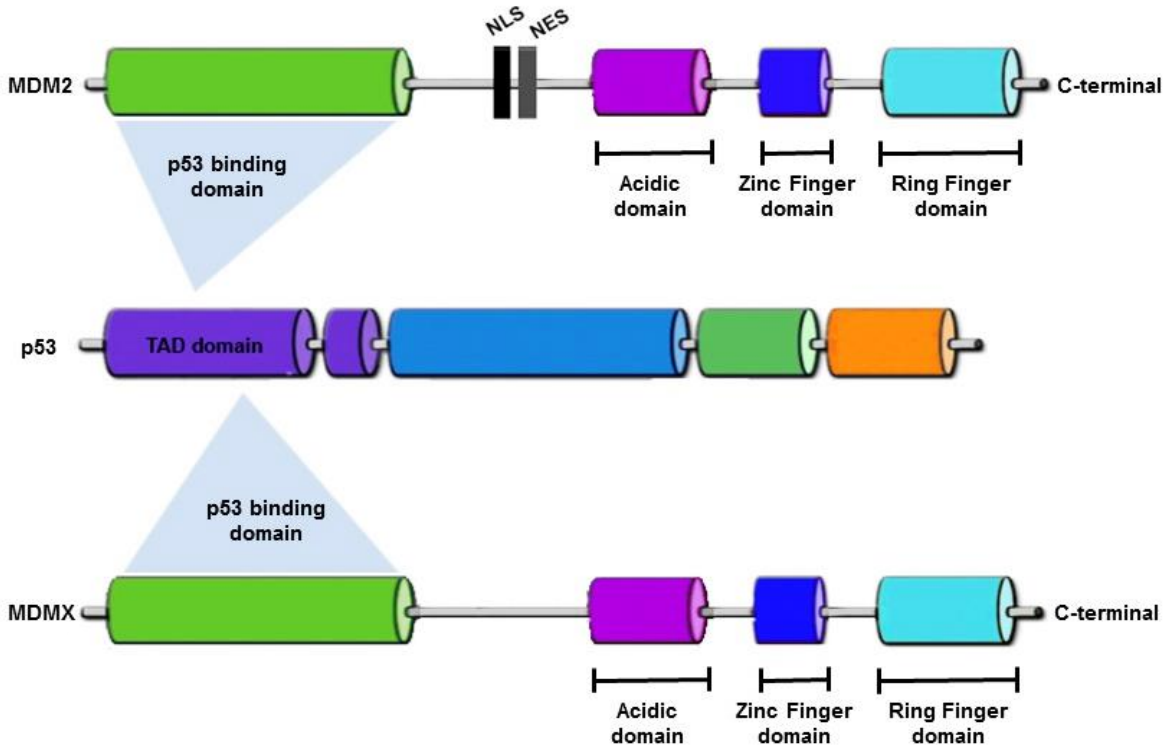

Figure 6. Schematic representation of the primary structure of MDM2 and MDMX proteins. Both MDM2 and MDMX proteins comprises a p53-binding domain, an acidic and a zinc-finger domain in their central portion followed by a *C*-terminal Ring-finger domain. Additionally, the MDM2 protein possess a nuclear localization signal (NLS) and a nuclear export signal (NES) sequence.

Several studies have reported the distinct but complementary roles of MDM2 and MDMX in p53 regulation. Indeed, although both proteins inhibit the p53 transcriptional activity, they have significant differences in their p53 regulation mechanisms. Previous *in vivo* studies confirmed the separated importance of this two proteins indicating that while MDM2 mainly regulates p53 stability, MDMX has a major role in regulating its activity [reviewed in (Toledo and Wahl, 2006; Toledo and Wahl, 2007; Vassilev, 2007)]. Interestingly, although the RING-finger domains of MDM2 and MDMX share a high level of homology, only MDM2 possess the ability to act as an E3 ubiquitin ligase, leading p53 and itself for degradation by ubiquitination (Marine and Jochemsen, 2005), whereas MDMX controls the levels of p53 by modulating the levels of MDM2 (Linke et al., 2008). Several studies, have shown that MDM2 and MDMX function in a non-overlapping manner, creating a negative feedback loop, which precisely controls the level and activity of p53 [reviewed in (Lenos and Jochemsen, 2011; Popowicz et al., 2011; Shadfan et al., 2012; Vassilev, 2007)]. Additionally, if in one way the endogenous negative regulators of p53 are able to inhibit and regulate its expression levels, p53 has shown to be capable to induce the transcription of both MDM2 [reviewed in

(Manfredi, 2010)] and MDMX proteins (Li et al., 2010), thus establishing a negative feedback loop (Figure 7).

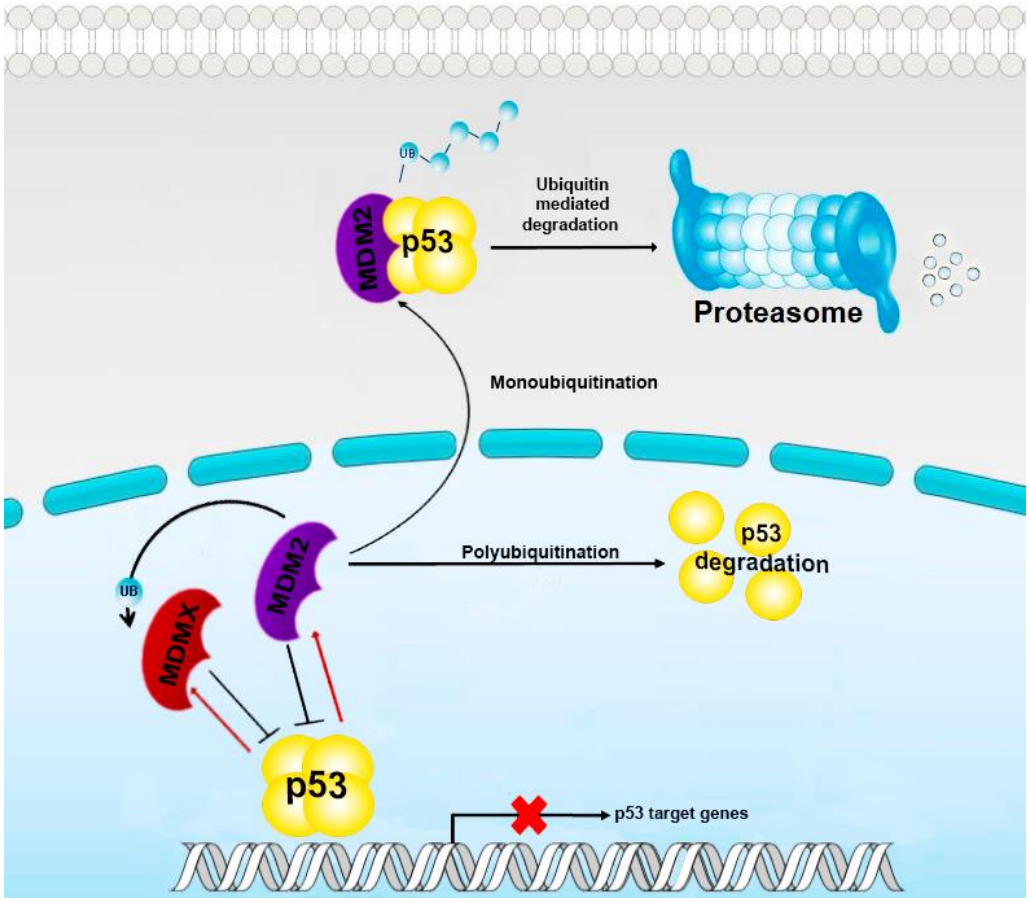

Figure 7. Regulation of p53 by its negative regulators MDM2 and MDMX. In normal cells, p53 increases the MDM2 and MDMX transcription over basal levels (red arrows). MDM2 and MDMX cooperate to inhibit the p53 function by modulating its transcriptional activity and by preventing its interaction with the general transcription machinery. As a homodimer, MDM2 is able to promote p53 nuclear export to the cytoplasm for ubiquitin-mediated degradation by the proteasome. Additionally, MDM2 can also form a heterodimeric complex with MDMX promoting the nuclear degradation of p53 (polyubiquitination) and MDMX ubiquitination by MDM2. These cellular mechanisms result in subtle control of p53 levels; (Ub: ubiquitin)

It has been described that, under normal conditions, MDM2 is able to inhibit the p53 activity through ubiquitination, either in the form of a homodimer or of a heterodimer, being the RING-finger domains integrity essential for these hetero or homodimerization (Lenos and Jochemsen, 2011; Linke et al., 2008). This complex results in the stabilization of MDM2 and, consequently, the ability of MDM2 to monoubiquitinate or polyubiquitinate p53 depends on its expression levels (Li et al., 2003). In fact, when the levels of MDM2 are low, monoubiquitination of p53 promotes the export of p53 from the nucleus to the cytoplasm

and consequent inhibition of p53 transcriptional activity (Figure 7). Once in the cytoplasm, p53 can be further ubiquitinated and degraded by p300. However, processes such as apoptosis has been described to be positively regulated by a number of different functions of cytoplasmic p53 [reviewed in (Hock and Vousden, 2014)]. Instead, high levels of MDM2 favour the p53 polyubiquitination, which results in the nuclear degradation of p53 (Figure 7). This degradation mechanism is responsible for the maintenance of low p53 levels in unstressed cells (Li et al., 2003).

Interestingly, the MDM2/X heterodimer complex induces polyubiquitination of p53, whereas MDM2 alone is primarily responsible for its monoubiquitination with further degradation of p53. Another consequence of MDMs heterodimerization is the MDMX ubiquitination (Figure 7) (Linke et al., 2008). The MDM2/MDMX E3 ligase as well as p53 can be regulated by deubiquitinases. Indeed, the deubiquitination and stabilization of both MDM2 and MDMX by the ubiquitin-specific protease 7(USP7) plays a pivotal role in the control of p53 stability [reviewed in (Hock and Vousden, 2014; Wang and Jiang, 2012)].

Many currently used cancer therapies, such as chemotherapy and radiation, have been primarily focused on p53 reactivation to trigger an apoptotic response. Unfortunately, high doses of these genotoxic treatments can also induce p53-independent pathways and thus may cause severe toxicities in normal tissues, which could eventually lead to secondary malignancies. In this context, efforts have been made in order to develop new selective and nongenotoxic inhibitors of the p53-MDMs interactions as an alternative to conventional cytotoxic chemotherapy [reviewed in (Shangary and Wang, 2009; Tisato et al., 2013)]. In fact, over the past years, the concept of selective chemotherapy has dominated the field of drug discovery and development. In this regard, the pharmacological restoration of the impaired function of p53, by disrupting its interaction with MDMs proteins has been shown to be an alternative therapeutic strategy against a broad spectrum of cancers with wt p53. Besides, the combination of various drugs that target multiple p53 pathways may be a useful strategy to achieve synergistic drug efficacy by reducing the genotoxic burden with the same or better anti-tumour effect [reviewed in (Popowicz et al., 2011; Zhao et al., 2013a)].

1.4.1 Inhibitors of MDM2 and MDMX in anticancer therapy

Over the past years, it has been difficult to develop small molecule inhibitors that disrupt large protein-protein interactions. Despite all the difficulties, and few progresses made in the first years, small molecules have been developed that compete for the p53 binding site of MDM2, inhibiting the binding and degradation of p53 by MDM2. The disruption of the p53-MDM2 interaction has shown to restore the wt p53 activity and to drive cancer cells

selectively into apoptosis [reviewed in (Shangary and Wang, 2009)]. Several studies of small molecule inhibitors of p53-MDM2 interaction, carried out in different cancer cell lines and animal models, support their usefulness as potential anticancer agents in tumours with overexpression of MDM2 [reviewed in (Vassilev, 2007)].

Several classes of chemical families have been reported as potent inhibitors of the p53-MDM2 interaction like the cis-imidazoline compounds, most commonly known by nutlins (Vassilev et al., 2004), the spiro-oxindoles (Zhao et al., 2013b), the isoindolinones (Hardcastle et al., 2011), the piperidinones (Rew et al., 2012), the xanthones (Leão et al., 2013a; Leao et al., 2013) and the oxazoloisoindolinones (Soares et al., 2014b), among others (Figure 8). A common way of action of several compounds, like nutlins and spiro-oxindoles, is mimicking the three pivotal amino acids that mediate the interaction of p53 with MDM2: *Phe19*, *Trp23* and *Leu26*, blocking the p53-MDM2 interaction (Zhao et al., 2013a). However, only few inhibitors of the p53-MDM2 interaction have advanced into clinical trials [reviewed in (Hoe et al., 2014)] namely RG7112, an oral formulation of nutlin series (Ray-Coquard et al., 2012; Vassilev et al., 2004), and MI-773 (spiro-oxindole) (Hoe et al., 2014; Zhao et al., 2013b).

Encouraged by the success in the design of small molecule inhibitors of the p53-MDM2 interaction, efforts have been made towards the development of small molecule inhibitors of the p53-MDMX interaction, such as SJ-172550 (Reed et al., 2010). Interestingly, high levels of MDMX protein are known to confer resistance to MDM2 inhibitors, which have shown very low binding affinity to MDMX. In fact, the apoptotic response of some p53-MDM2 interaction inhibitors, such as nutlin-3a, is markedly attenuated in MDMX-overexpressing tumours, such as in retinoblastomas and melanomas [reviewed in (Hoe et al., 2014)]. Since the simultaneous inhibition of the p53-MDM2 and p53-MDMX interaction is required for a full reactivation of p53, efforts have been made for the development of dual inhibitors of MDM2 and MDMX. Actually, very few compounds with dual specificity against both MDM2 and MDMX, namely pyrrolopyrimidine-based small molecules (Lee et al., 2011) and RO-5963 from the Indolyl hydantoin chemical family (Graves et al., 2012), were reported. Being these two compounds the only dual inhibitors found to date, the search for new ones is highly required.

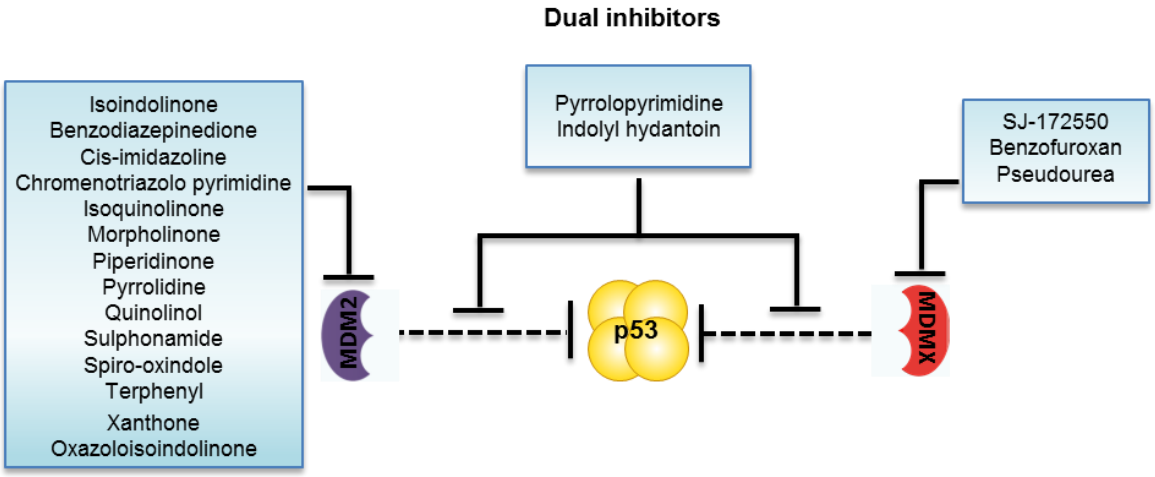

Figure 8. Schematic representation of small molecule inhibitors of MDM proteins. Isoindolinones, cis-imidazolines, xanthones, oxazoloisoindolinones, among others, act as inhibitors of the p53-MDM2 interaction. SJ-172550, benzofuroxan and pseudorea act as inhibitors of p53-MDMX interaction. The pyrrolopyrimidine-based small molecules and the compound RO-5963 from the Indolyl hydantoin chemical family, as shown to inhibit both p53-MDM2 and p53-MDMX interactions.

Inhibitors of the interaction of p53 with MDM2 and MDMX may also be important in cyclotherapy, in order to protect normal proliferating cells during antimitotic chemotherapy and offering to the patient a reduction in the side effects without loss of efficacy [reviewed in (Hoe et al., 2014; Popowicz et al., 2011; Zhao et al., 2013a)]. In this way, the pre-treatment with very low, non-genotoxic doses of MDM2/MDMX inhibitors will lead to cell cycle arrest in normal cells with functional p53, thus protecting them from the toxicity of the chemotherapy. This cytostatic effect of p53 can protect normal cells from the toxicity of S- or M-phase poisons. Conversely, tumour cells expressing mut p53, that will be insensitive to the MDM2 and MDMX inhibitors, will remain selectively vulnerable to the mitotic poison [reviewed in (Vassilev, 2007)]. One example of this applicability of MDM2 inhibitors was provided by Sur and colleagues, who showed in a mouse xenograft model that the neutropenia induced by the polo-like kinase 1 (PLK1) inhibitor, BI-2536, could be prevented by the prior exposure to nutlin-3a, reducing the toxicity associated with BI-2536. Importantly, this MDM2 inhibitor have no impact on the antitumour effect of this PLK1 inhibitor (Sur et al., 2009).

Additionally, several recent studies have investigated the possible synergistic interactions between traditional drugs and nutlin-3a. Indeed, nutlin-3a combined with cisplatin, methotrexate or doxorubicin has been reported to induce apoptosis much more efficiently than either drug alone in cells with dysfunctional p53 pathways. Actually, nutlin-3a seemed

to potentiate the effect of these drugs with simultaneous reduction of their cytotoxicity (Mir et al., 2013; Ohnstad et al., 2011).

1.5 p53 mutant forms

Mutations in *TP53* allow tumour cells to subvert the biological meaning of the p53 pathway turning it into a tumour promoting network. It is important to recognize that not all p53 mutants are equal, not merely as a conceptual distinction, but also for the practical implication. Nowadays the objective is to personalize cancer therapy as much as possible, so it is important not only to differentiate wt and mut p53, but also to ascertain the particular mutation that a patient´s tumour bears. Over the last years, p53 mutants have been found to actively contribute to tumour proliferation, survival, limitless replication, somatic cell reprogramming, genomic instability, inflammation, disruption of tissue architecture, migration, invasion, angiogenesis, and metastasis. Amazingly, this places mut p53 in a central role in tumourigenesis, impacting nearly all of the 10 cancer hallmarks (Figure 1) [reviewed in (Freed-Pastor and Prives, 2012; Girardini et al., 2014)].

The p53 function is almost always compromised in tumour cells, usually as a result of somatic mutations, which occurs in approximately half of all human cancers. Nevertheless, depending on the type of cancer, the frequency of *TP53* mutations is highly variable ranging from less than 5% (in cervical carcinoma) to 90% (in ovarian carcinoma) [reviewed in (Leroy et al., 2014)].

Whereas somatic *TP53* mutations contribute to sporadic cancer, germline mutations cause a rare type of cancer predisposition, known as Li–Fraumeni Syndrome. This unique syndrome, among cancer predispositions, results from an inherited point mutation in a conserved region of p53 and it is not associated with site-specific tumours, but rather with a variety of tumour types occurring at a relatively early age (Malkin et al., 1990; Srivastava et al., 1990). Importantly, both somatic and germline *TP53* mutations are usually followed by loss of heterozygosity during tumour progression, which suggests that a selective force inactivates the remaining wt allele [reviewed in (Brosh and Rotter, 2009)].

Structural studies of p53 have revealed that the majority (around 75%) of p53 mutations found in human cancers are single amino acid substitutions (missense mutations). These missense mutations lead to the production of a mut protein that differs from the wt p53 by one amino acid, resulting in the formation of a full-length protein incapable of activate p53 target genes and suppress tumourigenesis, and that consequently leads to an accumulation of mut p53 in human tumour cells [reviewed in (Brosh and Rotter, 2009; Freed-Pastor and Prives, 2012)].

Interestingly, the majority of these mutations occurs in the genomic region encoding the sequence-specific DBD of p53, leading to structural changes that destabilizes the p53 structure and disrupt the proper conformation of the sequence-specific DBD. Therefore, these mutations can abrogate the sequence-specific transactivation ability of wt p53 [reviewed in (Bassett et al., 2008; Ozaki et al., 2013)].

Although mutations have been found in almost every codon within the DBD of p53, only the most frequently occurring mutations have been deeply studied for their contribution to cancer progression. The existence of these hotspot residues can be explained both by the susceptibility of particular codons to carcinogen-induced alterations and by positive selection of mutations that render the cell more susceptible to growth and survival [reviewed in (Freed-Pastor and Prives, 2012; Muller and Vousden, 2014)]. In fact, *TP53* mutations in cancer affect manly six "*hotspot*" residues within the DBD, namely the residues R175, G245, R248, R249, R273 and R282 [reviewed in (Muller and Vousden, 2014; Ozaki et al., 2013)]. Furthermore, there are two other residues of p53 with a significant mutation incidence, namely Y220 and R280 (Soussi and Béroud, 2012) (Figure 9). For their relevance at the present work, mutations at residues R175, Y220, R273 and R280 will be described in greater detail.

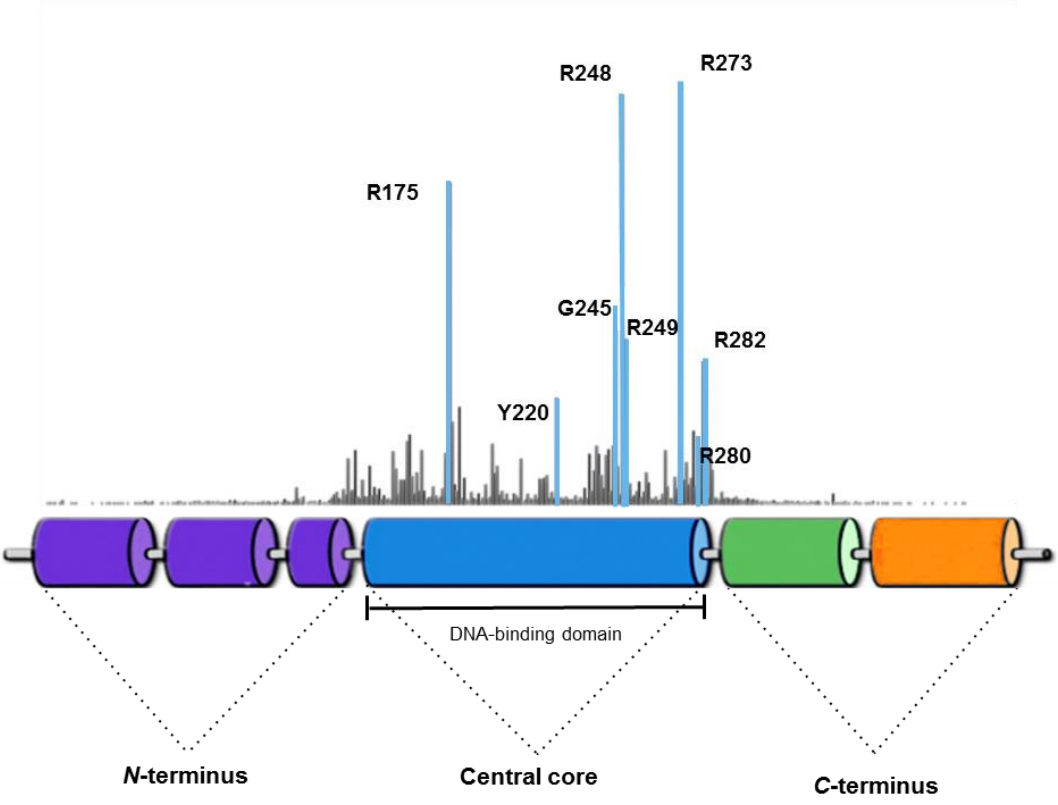

Figure 9. Schematic representation of the prevalence of *TP53* missense mutations in human cancers. The columns indicate the relative frequency of cancer-associated mutations for each residue according to the *TP53* mutation database of *The p53 Web Site* [adapted from (Millau et al., 2009)].

A well-established classification system for mut p53 is based on its role as a transcriptional factor. Thus, according to the effect of the amino acid substitution, p53 mutants can generally be classified into two main categories: ‘‘conformational / structural’’ or ‘‘DNA contact’’ mutants. Both types are missense mutations that prevent the pro-apoptotic p53 transactivation functions by blocking the binding of p53 with target promoter sites on DNA. The DNA contact mutants [such as p53 R273H and p53 R280K (Cho et al., 1994)] prevent the wt transcriptional activity without dramatically affecting the conformation of p53 protein. However, the majority of mut p53 proteins found in human tumours are structural mutants [p53 R175H (Cho et al., 1994) and p53 Y220C (Boeckler et al., 2008)] that disrupt the structure of the p53 protein, on either a local or global level (Liu et al., 2013; Yu et al., 2012).

These missense mutations usually confer to the mut protein a dominant-negative activity over the remaining wt allele, in this way, when wt and mut p53 alleles exist in a heterozygous status in tumour cells, mut p53 can block the function of wt p53 (Figure 10). Mutant proteins usually accumulate in large amounts in cancer cells, which is partially explained by a loss of the wt p53 function, including a loss of the ability to induce the transcriptional activation of its negative regulator MDM2 [reviewed in (Brosh and Rotter, 2009)] (Figure 10). Interestingly, it has been well-documented that some tumour-associated mut p53 not only lose the tumour suppressive function of wt p53 and acquire dominant-negative activities, but also gain new oncogenic functions that are entirely independent of wt p53, referred as the gain-of-function (GOF) (Figure 10) [reviewed in (Bassett et al., 2008; Girardini et al., 2014)]. Thus, the GOF of mut p53 can contribute to the close correlation between the expression of mut p53 and the higher frequency of tumour development, chemoresistance, angiogenesis, migration, increased invasive and metastatic potential and poor prognosis of cancer patients [reviewed in (Freed-Pastor and Prives, 2012)]. Indeed, previous studies have proposed that acquisition of oncogenic GOF activities, were clearly demonstrated in two mut p53 knock-in mouse models of Li-Fraumeni syndrome. These models have reported evidence of GOF for R175H and R273H mutants (Lang et al., 2004; Olive et al., 2004). Recent data suggests that the activity of p53 GOF mutants may also be mediated by inhibitory interactions with other transcriptional factors that normally bind to gene promoters [reviewed in (Song and Xu, 2007; Xu, 2008)]. An important example is the interaction of such p53 mutants with the family members p63 and p73 [reviewed in (Brosh and Rotter, 2009)].

Interestingly the core domain of p53 is only marginally stable at body temperature. Hence, any mutation that reduces the thermodynamic stability of the protein will have a profound effect on the amount of folded protein in the cell [reviewed in (Bullock and Fersht, 2001)]. DNA-contact mutants, such as R273H, have only a small effect on the thermodynamic

stability of the protein. Instead, structural "*hotspot*" mutations in the DNA-binding surface lead to thermodynamic instability, being functional at low temperature but denature at higher temperatures [reviewed in (Butler and Loh, 2005; Friedler et al., 2003)]. Several p53 mutants reduce the melting temperature of p53, leading to its fast degradation. Indeed, R175H and Y220C are examples of p53 mutants dependent on temperature (Gaiddon et al., 2001). Highly destabilized mutants unfold more than 10 times faster than the wt core domain, whereas the contact mutants have roughly the same half-life as the wt (Butler and Loh, 2005; Friedler et al., 2003).

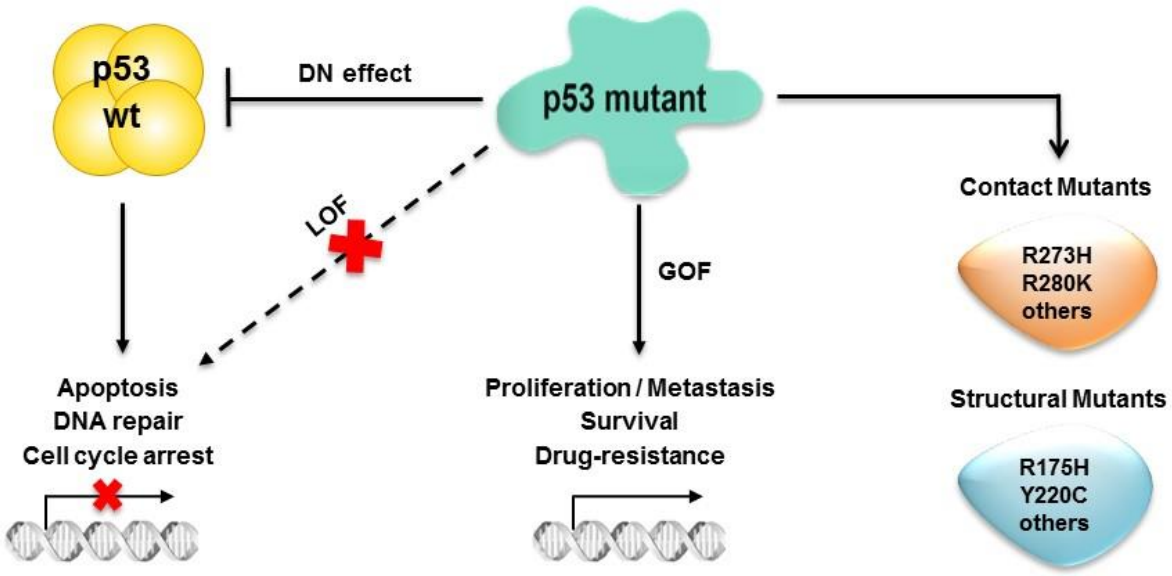

Figure 10. Functional impact of *TP53* mutations. The phenotypic effects of p53 mutation can usually confer three non-mutually exclusive characteristics: a dominant negative (DN) effect of mut p53 over the wt protein, a loss of function (LOF) and a gain of function (GOF). The GOF plays a role in both contact (such as R273H and R280K) and structural mutants (such as R175H and Y220C) [adapted from (Brosh and Rotter, 2009)]

Both R175H and R273H mutations (two of the six *hotspot* mut p53) present a change from arginine to histidine amino acids and R280K has a change from arginine to lysine amino acids. In the DNA contact mut R273H and R280K, the substitution has little or no effect on p53 folding, but directly affects its binding to DNA, while in the structural mut R175H the substitution leads to destabilization and conformational perturbations of the protein that interfere with its oligomerization and DNA-binding, resulting in more severe changes in the protein structure (Vrba et al., 2008).

Y220C, the ninth most frequent mutation found in human cancers (Figure 9), shows a substitution of the tyrosine by cysteine, which leads to a decrease of the thermodynamic stability of p53 [reviewed in (Girardini et al., 2014)]. Thus, a druggable cavity is created on

the surface of the protein at the site of mutation that is particularly suited for pharmacological manipulation (Boeckler et al., 2008).

Finally, mutations at the residues R280K, Y220C, R273H and R175H have been associated with GOF activities (Figure 10) (Fontemaggi et al., 2009; Kang et al., 2013; Lang et al., 2004; Olive et al., 2004).

1.5.1 Reactivation of wild-type function to mutant p53

Due to the high frequency of p53 mutations in cancer and to the increased resistance of mutant p53-expressing tumours to conventional chemo- and radiotherapy, the restoration of wt conformation and activity is a promising strategy for cancer therapy. However, based on the conventional drug development process, p53 is not an ideal therapeutic target, since it is neither an enzyme nor a receptor. Despite the difficulties associated with the development of drugs targeting p53, recent technological advances, in association with the knowledge about p53 obtained over the past 30 years, made the development of tumour-specific p53 restoration therapies a realistic ambition [reviewed in (Brosh and Rotter, 2009; Chen et al., 2010; Farnebo et al., 2010)].

Several strategies have been designed to restore the impaired function of wt p53 in its mut forms, including the introduction of second-site mutations, antibody binding, short peptides and small molecules [reviewed in (Selivanova and Wiman, 2007)]. The rescue strategies vary depending on the mutation types. So, in tumours harbouring a DNA-contact mut, the attempts were to introduce functional groups that create new contacts or stabilize the scaffolding position in the remaining contact sites. On the other hand, the functions of conformation mutants can be restored by a synthetic peptide [reviewed in (Bassett et al., 2008; Wang and Sun, 2010)] derived from the p53 *C*-terminal region that, when introduced into tumour cells harbouring mut p53, leads to the induction of p53-regulated genes and apoptosis, which results in the stabilization of the wt conformation of the mut protein (Selivanova et al., 1997).

More recently, attempts have been made to identify small molecules that reactivate mut p53. These molecules should directly interact with mut p53 DBD leading to the restoration of wt folding and, consequently, of DNA-binding and transactivation activity to mut p53. This strategy led to the identification of small molecules that restore the wt p53 function to several p53 mutants. Screening studies have identified small molecules, including CP-31398 (Foster et al., 1999), ellipticine (Shi et al., 1998), PRIMA-1 (Bykov et al., 2002), MIRA-1 (Bykov et al., 2005) and RITA (Grinkevich et al., 2009) as reactivating agents of mut p53.

The first reported small molecule, CP-31398, allows mut p53-expressing cells to restore the protein folding of mutated p53 to a more natural conformation that allows a wt function and even impair tumour growth (Table 1) (Foster et al., 1999). Despite this promising start, it was later discovered that CP-31398 does not bind to the p53 core domain and does not stabilize p53 *in vitro*, but instead it is a DNA intercalator (Rippin et al., 2002).

Of the small molecules that target mut p53, the most well studied so far has been PRIMA-1 (p53-Reactivation and Induction of Massive Apoptosis), which has been shown to reactivate missense mut p53. This compound aims to restore sequence-specific DNA binding and change the mut p53 conformation to wt, leading to the transactivation of p53 target genes and thus halt tumour growth. PRIMA-1 rescued both DNA contact and structural p53 mutants (Table 1) (Bykov et al., 2002; Lambert et al., 2009). A more potent methylated structural analog of PRIMA-1, APR-246/PRIMA-1^{Met}, has advanced to the clinical trials, and the phase-I evaluation has been recently completed (Table1) [reviewed in (Hoe et al., 2014)]. In contrast to CP-31398, PRIMA-1 does not activate wt p53 (Lambert et al., 2009).

While the above-mentioned compounds target multiple p53 mutants, one of the most impressive approaches from recent years has been the development of a structure-based ‘‘mutant-specific’’ drug, PhiKan083. PhiKan083 is a carbazole derivative found from *in silico* screening of the crystal structure of p53. PhiKan083 binds to a unique pocket in the core domain of p53-Y220C, stabilizing the conformation of the core domain and restoring the transactivation of p53 target genes. By binding mutated p53, PhiKan083 can raise the melting temperature of the mut p53-Y220C, and the rate of thermal denaturation subsequently slows down, resulting in the reactivation of p53 function (Table 1) (Boeckler et al., 2008; Rauf et al., 2013). A new compound recently developed, PK7088, has also shown to bind to Y220C core domain reactivating cellular p53 functions (Liu et al., 2013).

The small molecule NSC319726 has shown to favour a wt conformation, inducing the transcription of wt p53 target genes and apoptosis in cell lines harbouring a R175H mutation. Moreover, this compound selectively inhibited xenograft tumour growth of R175H-mut p53 cells (Yu et al., 2012) (Table 1).

Another small molecule, SCH529074, was identified in an *in vitro* DNA-binding assay. This compound binds to the DBD of mut p53 working as a chaperone, restoring proper folding and DNA-binding activity to mut p53. SCH529074 restores the activity to both DNA contact (R273H) and structural (R249S) p53 mutants (Demma et al., 2010) (Table 1).

Table 1. Small molecule reactivators of mut p53 through restoration of wt p53 conformation.

Compound	Mutant p53	*In vivo* anti-tumour activity	Genotoxicity	Pre-clinical Toxicity	Clinical trials	Ref
CP-31398	R175H R280K R273H	Yes	Yes	Yes	Data not available	(Foster et al., 1999; Rippin et al., 2002)
Prima-1	R175H R273H R280K	Yes	No	No	Data not available	(Bykov et al., 2002; Lambert et al., 2009)
PRIMA-1^{Met} (APR-246)	R175H R273H	Yes	No	No	Phase I/II	(Bykov and Wiman, 2014; Lambert et al., 2009)
NSC319726	R175H	Yes	Data not available	No	Data not available	(Yu et al., 2012)
Phikan 083	Y220C	Data Not available	Data not available	Data not available	Data not available	(Boeckler et al., 2008)
SCH529074	R273H R249S	Yes	Data not Available	Data not available	Data not available	(Demma et al., 2010)

1.6 Yeast model in the study of p53 related proteins

The high degree of conservation of cellular processes and molecular pathways with human cells has made the yeast *Saccharomyces cerevisiae* a powerful model to study human proteins and the molecular mechanisms underlying the pathobiology of several human diseases. Among all eukaryotic organisms, the yeast cell model presents many advantages including easy and low-cost manipulation, short generation time and compliance to genetic manipulation [reviewed in (Mager and Winderickx, 2005; Pereira et al., 2012a)].

S. cerevisiae has greatly contributed to the elucidation of basic cellular processes related to cell cycle and cell death, among others [reviewed in (Khurana and Lindquist, 2010)]. However, it must be noted that as a unicellular organism some genes involved in human disease may not be present in the yeast genome and several aspects of human disorders, which rely on multicellularity and cell-cell interactions, are difficult to evaluate. Interestingly, if the gene implicated in the human disease is conserved in yeast, its function can be directly studied. Moreover the heterologous expression of human proteins in yeast (commonly referred to as “humanized yeast”) has brought us the possibility for an independent analysis of a wide range of human proteins. However, if the gene implicated in the human disease has no orthologues in yeast, its heterologous expression will allow the study of the human protein in a simpler eukaryotic environment, without the interference of other proteins with similar functions, endogenous regulators and redundant processes, which can be very

advantageous [reviewed in (Pereira et al., 2012a; Pereira et al., 2012b)]. This is the case of p53 and its negative regulators MDM2 and MDMX, for which no orthologues has been identified in yeast, making possible the study of the activity of individual human p53 proteins heterologously expressed in this microorganism [reviewed in (Pereira et al., 2012a; Smardová et al., 2005)].

In fact, over the recent years, several yeast-cell based assays have been developed for functional, molecular and pharmacological studies of p53 family proteins.

The yeast two-hybrid (Y2H) assay is the most known and used assay to study interactions between proteins. In this assay, the protein of interest is fused to the DBD of a transcription factor, such as *Gal4*, while the potential interactor protein is fused to a transactivation domain (Figure 10B). Upon co-expression, protein-protein interaction is detected because association of the two domains of transcription factor leads to the expression of a reporter gene (Ratushny and Golemis, 2008). There are many studies that explore the interaction of p53 family proteins with other proteins using the Y2H assay. For example, Freedman and colleagues performed a modified Y2H assay, in order to determine the amino acids of MDM2 that are critical for binding to p53. This method was important to reveal not only that the interaction between the anti-apoptotic protein translationally control MDM2 protein and p53, but also to find the critical binding sites between these two proteins (Freedman et al., 1997). Later, Sharp and colleagues, using an Y2H screening protocol to identify other factors with a potential role in influencing the expression and/or function of MDM2, reported the physical interaction of MDM2 with MDMX (Sharp et al., 1999). The Y2H assay was also used to study interactions between members of the p53 family (Kaghad et al., 1997) and mutants (Ratovitski et al., 2001). In 1996, Vidal and colleagues developed a reverse Y2H assay applicable for drug screening, in which cells grow when the interaction between proteins does not occur due to drugs. The reverse Y2H assay is an excellent assay to find compounds that inhibit unwanted protein interactions, like inhibitors of p53-MDM2 interaction (Vidal et al., 1996).

Ishioka and colleagues described one of the first yeast cell-based assays to study the p53 family of proteins, the Functional Analysis of Separated Alleles in Yeast (FASAY) assay. This technique consists in the amplification of the p53 cDNA, obtained from tumour samples or other tissues, by reverse transcriptase PCR (RT-PCR) and in the co-transformation of a yeast reporter strain with the PCR product and a gapped expression plasmid. The p53 status is evaluated using plates without histidine. Yeast clones expressing a functional p53 are His+ due to the capacity to express the HIS3 reporter gene and will therefore grow; yeast clones expressing a mutated p53 are His- and will not grow. In this way, the FASAY assay, consists of a functional analysis of the separated alleles of *TP53* in yeast in order to

evaluate the p53 activity in cancer (Ishioka et al., 1993). Using an improved version of this cell system, it was verified that some *TP53* mutations led to the partial loss of the p53 transactivation function (Kovvali et al., 2001).

More recently it was developed the dual-luciferase functional assay (Figure 11A), which uses a luciferase reporter gene containing a p53 response element (RE) placed upstream of a minimal promoter (pCYC1). In this way, when wt p53 is activated, it will bind to the p53 RE, inducing the transcription of the luciferase reporter gene and the synthesis of luciferase. Therefore, the p53 transcriptional activity is evaluated by the quantification of the light emitted, which is directly proportional to the luciferase oxidative activity. This dual-luciferase transactivation assay was adapted to the high throughput screening (HTS) of co-factors and small molecules that can influence the p53 network (Andreotti et al., 2011). In spite of all the advantages of this yeast-based assay, it is an artificial system, since it relies on the insertion of a human reporter gene containing a p53 RE in yeast cells, and an expensive cell system for HTS.

During the last years, our research group has been focused on the development and validation of yeast target-based screening assays, for pharmacological research directed to distinct cancer-related human proteins, such as protein kinase C (PKC), caspases and p53 family proteins [reviewed in (Gomez-Casati et al., 2012; Pereira et al., 2012a; Pereira et al., 2012b)].

In our first work with p53, it was confirmed that expression of wt p53 in yeast induced a marked growth inhibition associated with S-phase cell cycle (Coutinho et al., 2009). This toxic effect of p53 in yeast allowed the development of a yeast phenotypic assay, based on simple measurements of cell growth. Additionally, this yeast growth-inhibitory assay was used as a screening tool to search for modulators of p53 activity. In subsequent studies, it was shown the conservation in yeast of the inhibitory effect of MDM2 and MDMX on p53 activity. In fact, as in mammalian cells, when co-expressed wt p53 with both MDM2 and MDMX markedly reduce the p53 activity in yeast leading to the reduction of the p53-induced yeast growth inhibition and cell cycle arrest (Leão et al., 2013a; Leão et al., 2013b). It was also found that the p53-induced yeast growth inhibition and cell cycle arrest, are associated with a marked increase of actin protein levels, when compared with the control yeast (Figure 10C). Moreover, it was shown that the wt p53-induced actin protein levels are modulated by natural (MDM2 and MDMX) and chemical (nutlin-3a and SJ-172550) regulators of p53 activity. Furthermore, wt p53 could stimulate transcription from a minimal promoter containing a fragment of the *ACT1* upstream sequence. Thus, *ACT1* is proposed as a putative endogenous p53 target gene (Figure 10C) (Leão et al., 2013b).

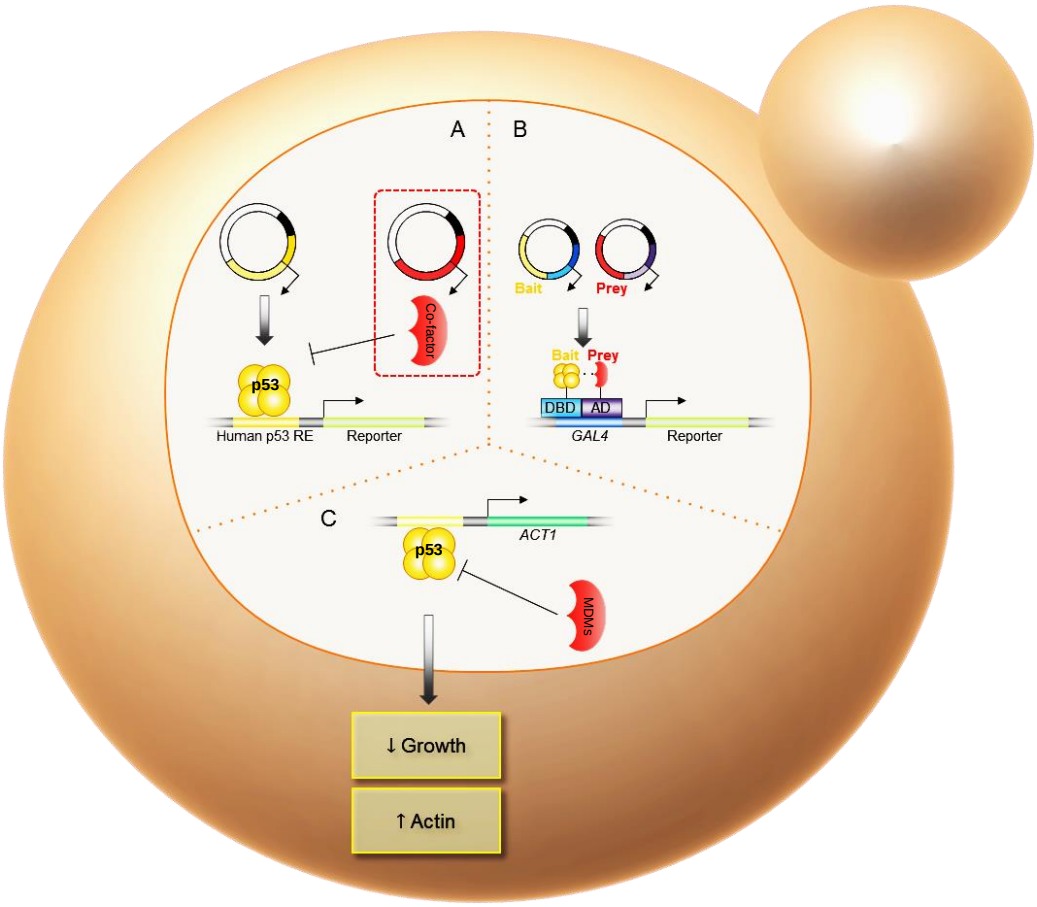

Figure 11. Schematic representation of three yeast models to study p53. A) **Yeast-based p53 dual-luciferase transactivation assay** expressing human wt/mut p53 or co-expressing human wt p53 and a co-factor (for example MDM2). The p53 transcriptional activity is evaluated by quantification of the luciferase activity, which is directly proportional to the light units measured in a plate reader. **B**) **Yeast two-hybrid assay,** in which the coding sequence of protein X (bait) is fused to the *GAL4-DBD* and the coding sequence of protein Y (Prey) is fused to the *GAL4-AD*. With the interaction of the two proteins X and Y, the functionality of the transcription factor is reconstituted, *GAL4* binds to its specific *Upstream Activation Sequence* leading to the transcription of the reporter gene. **C) Yeast p53-MDM2/p53-MDMX phenotypic assay**. The expression of wt p53 induces growth inhibition and increases the actin protein levels, which is reverted by MDM2 and MDMX.

Using this yeast phenotypic assay, we were able to search for new inhibitors of the p53-MDM2 and p53-MDMX interactions. In fact, the use of this cell system led to the identification of new small molecules inhibitors of the p53-MDM2 interaction such as the compound with a xanthone scaffold (LEM1) (Leão et al., 2013a), two other xanthone derivatives, the α-mangostin and gambogic acid (Leao et al., 2013) and, more recently, a compounds with an oxazoloisoindolinone scaffold (Soares et al., 2014b).

Despite some limitations, the yeast screening assay as proved to be effective in a first-line screening of potential active compounds, reducing the costs and expediting the discovery of new drugs.

1.7 Aims of research

The main objectives of this thesis were to:

i) Use a previously developed yeast target-directed screening approach to search for new small molecule inhibitors of the p53-MDM2/MDMX interaction;

ii) Validate in human tumour cell lines the molecular mechanism of action of potential dual inhibitors of the p53-MDM2/MDMX interaction identified in yeast;

iii) Develop a new yeast target-directed screening assay to search for reactivators of some of the most prevalent human mut p53 forms, namely R280K, R175H, R273H and Y220C.

Chapter 2

OXAZ-1: a new small molecule with *in vitro* antitumour activity through selective activation of a mitochondria-mediated p53 pathway and potential MDM2/MDMX inhibition

2 Introduction

MDM2 and MDMX are two major factors that contribute to cancer development due to the inactivation of wt p53. Disruption of the p53-MDMs regulatory network has therefore clear implications for tumorigenesis and presents exciting opportunities for cancer therapy (Wade et al., 2013). The antitumour activity of small molecule inhibitors of the p53-MDMs interaction has already been confirmed in preclinical studies and early-phase of clinical trials. However, to date, most of the current efforts have been focused on the p53-MDM2 interaction (Li and Lozano, 2013; Wade et al., 2013). The role of MDMX in the fine regulation of p53 is still emerging, but it is well-established that even normal levels of MDMX can partially silence activated p53. Additionally, MDMX overexpression (namely in retinoblastomas and melanomas) renders cancer cells highly resistant to MDM2-only inhibitors, such as nutlin-3a, since they are unable to liberate p53 from MDMX (Graves et al., 2012; Li and Lozano, 2013). This clearly indicates that dual inhibition of MDM2 and MDMX may substantially improve the outcome of this p53 activation strategy due to the full p53 activation (Li and Lozano, 2013; Wade et al., 2013). In spite of this, recent efforts to develop this type of molecules have been hindered by structural differences in the p53 pockets of MDM2 and MDMX (Riedinger and McDonnell, 2009). In fact, only recently, the small molecules based on a pyrrolopyrimidine scaffold (Lee et al., 2011) and RO-5963 (Graves et al., 2012) were identified as dual inhibitors of MDM2 and MDMX.

In this chapter, we report the identification of a potential dual inhibitor of the p53 interaction with MDM2 and MDMX, the *N*-tosylindole OXAZ-1, from the screening of a small library of tryptophanol-derived oxazolopiperidone lactams, using a yeast assay. The identification of this selective activator of the p53 pathway may open the way to the development of a new class of dual MDM2/MDMX inhibitors with promising applications in anticancer therapy.

2.1 Material and methods

2.1.1 Compounds

OXAZ-2, OXAZ-3, OXAZ-4, OXAZ-5, and OXAZ-6, whose molecular structures are shown in Figure 11 were synthesized according to the described procedures (Amat et al., 2007a; Amat et al., 2007b; Pereira et al., 2014).

Nutlin-3a was from Alexis Biochemicals (Grupo Taper, Sintra, Portugal); doxorubicin and SJ-172550 were from Sigma-Aldrich (Sintra, Portugal); etoposide was from Calbiochem (VWR, Carnaxide, Portugal). All tested compounds were dissolved in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Sintra, Portugal).

2.1.2 Synthesis of OXAZ-1

A solution of tryptophanol-derived oxazolopiperidone lactam OXAZ-5 (1 eq.) in CH_2Cl_2 was cooled to 0°C. p-Toluenesulphonyl chloride (1.2 eq.) and tetrabutylammonium chloride (cat., 10% mmol) were added and the mixture was stirred for 10 min. Then, an aqueous solution of NaOH (30% m/v, 0.5 v CH_2Cl_2) was added and the reaction was allowed to stand at room temperature for 24 h. After this period, the reaction was diluted with CH_2Cl_2 and the phases were separated. The organic phase was washed with HCl (1M) and dried over Na_2SO_4. After solvent evaporation the crude compound was purified by flash chromatography (EtOAc/n-hexane 1:1). The desired compound was recrystallized from dichoromethane. 1H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 8.1 Hz, 1H), 7.72 (m, 3H), 7.36 (s, 1H), 7.31 (t, J = 7.7 Hz, 1H), 7.25 (m, 1H), 7.21 (m, 2H), 4.70 (dd, J = 9.9, 2.5 Hz, 1H), 4.23 (m, 1H), 3.95 (d, J = 9.4 Hz, 1H), 3.74 (t, J = 7.8 Hz, 1H), 3.70 (s, 3H), 3.58 (d, J = 14.1 Hz, 1H), 2.68 (dd, J = 14.0, 9.8 Hz, 1H), 2.60 (dd, J = 17.6, 4.6 Hz, 1H), 2.37 (m, 3H), 2.33 (s, 3H), 2.28 (d, J = 12.0 Hz, 1H), 2.10 (dd, J = 17.6, 10.2 Hz, 1H), 1.21 (m, 1H). Elemental Anal. calcd. for $C_{26}H_{28}N_2O_6S$: C 62.89, H 5.68, N 5.64, found: C 62.98, H 5.84, N 6.00. The 1H NMR spectra was found to be identical to the one described in (Amat et al., 2007b).

2.1.3 Plasmids

The yeast expression vectors were used: pGADT7-(*LEU2*) encoding human MDM2 (kindly provided by Dr Xue-Min Zhang; National Center of Biomedical Analysis, China) or MDMX (kindly provided by Dr Martin Scheffner; University of Konstanz, Germany) under *ADH1* constitutive promoter; pLS89-(*TRP1*) encoding human wt p53 under *GAL1-10* inducible promoter (kindly provided by Dr Richard Iggo; Swiss Institute for Experimental Cancer Research, Switzerland).

2.1.4 Yeast target-directed screening assay

S. cerevisiae (strain CG379) expressing human wt p53 alone and combined with human MDM2 or MDMX were obtained in previous works (Leão et al., 2013a; Leão et al., 2013b). For expression of human proteins, cells (routinely grown in minimal selective medium) were diluted to 0.05 OD_{600} in selective induction medium containing 2% (w/w) galactose, 1% (w/w) raffinose, 0.7% (w/w) yeast nitrogen base without amino acids from Difco (Quilaban, Sintra, Portugal) and all the amino acids required for yeast growth (50 mg/mL) except leucine and tryptophan. Yeast cells were incubated at 30 ºC under continuous orbital shaking (200 r.p.m.) with 0.1 – 50 μM compounds or 0.1% DMSO only, for approximately 42 h (time required by control yeast, co-transformed with the empty vectors pLS89 and

pGADT7 incubated with DMSO only to achieve 0.4 OD_{600}). Yeast growth was analysed by counting the number of colony-forming units (CFU) after 2 days incubation at 30 ºC on Sabouraud Dextrose Agar from Liofilchem (Frilabo, Porto, Portugal).

2.1.5 Human tumour cell lines and growth conditions

The human colon adenocarcinoma HCT116 cell line harbouring a wt p53 form (HCT116 $p53^{+/+}$), and its isogenic derivative in which p53 has been knocked out (HCT116 $p53^{-/-}$), and the human breast adenocarcinoma MCF-7 tumour cell line were used. Cell lines were routinely cultured in RPMI-1640 with ultraglutamine medium from Lonza (VWR, Carnaxide, Portugal) supplemented with 10% fetal bovine serum from Gibco (Alfagene, Carcavelos, Portugal) and maintained in a humidified incubator at 37 °C with 5% CO_2 in air.

2.1.6 Sulforhodamine B (SRB) assay

For the analyses of the effect of compounds on the *in vitro* growth of human tumour cell lines, cells were plated in 96-well plates at a final density of 5.0×10^3 cells/well and incubated for 24 h. Cells were then exposed to serial dilutions of compound (from 1.85 to 150 µM). The effect of the compounds was analysed following 48 h incubation, using the SRB assay. Briefly, following fixation with 10% trichloroacetic acid, plates were stained with 0.4% SRB, both from Sigma-Aldrich (Sintra, Portugal), and washed with 1% acetic acid. The bound dye was then solubilized with 10 mM Tris Base and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc., Synergy MX, USA). The solvent of the compounds (DMSO) corresponding to the maximum concentration used in these assays (0.25%) was included as control. The concentration of the compound that causes a 50% reduction in the net protein increase in cells during treatment (GI_{50}, growth inhibition of 50%) was determined for all tested compounds.

2.1.7 Analysis of cell cycle and apoptosis in human tumour cell lines

HCT116 $p53^{+/+}$ and MCF-7 cells were plated in 6-well plates at a final density of 1.5×10^5 cells/well. After 24 h incubation, cells were treated with the GI_{50} and with twice the GI_{50} concentration ($2xGI_{50}$) of OXAZ-1 or DMSO only for 24 h. For cell cycle analysis, cells were thereafter fixed in ice-cold 70% ethanol and incubated at 37 ºC with RNase A from Sigma-Aldrich (Sintra, Portugal) at a final concentration of 20 µg/mL for 15 min, and further incubated with 50 µg/mL propidium iodide (PI) from Fluka (Sigma-Aldrich, Sintra, Portugal) for 30 min, followed by flow cytometric analysis. For apoptosis analysis, cells were analysed

by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit I from BD Biosciences (Enzifarma, Porto, Portugal), according to the manufacturer's instructions.

2.1.8 Western blot analysis

To prepare whole protein extracts from yeast, these cells were lysed with Cellytic™ Y Cell Lysis Reagent containing EDTA-free protease inhibitor cocktail from Sigma-Aldrich (Sintra, Portugal). To prepare the whole protein extracts from human tumour cell lines, cells were lysed with RIPA buffer containing EDTA-free protease inhibitor cocktail from Sigma-Aldrich (Sintra, Portugal). For mitochondrial and cytosolic fractions of human tumour cell lines, the Mitochondrial Fractionation Kit from Active Motif (Frilabo, Porto, Portugal) was used according to the manufacturer's instructions.

Whole protein extracts were quantified using the Coomassie staining Bradford from Sigma-Aldrich (Sintra, Portugal). Proteins (40 µg) were electrophoresed using a 10% SDS-PAGE and transferred to a Whatman nitrocellulose membrane from Protan (VWR, Carnaxide, Portugal). Membranes were blocked with 5% milk and probed with a mouse monoclonal anti-p53 (DO-1), anti-MDM2 (D-12), anti-Bax (2D2), anti-PUMA (B-6), anti-PARP (C2-10) and anti-cytochrome c (A-8) followed by an anti-mouse horseradish-peroxidase (HRP)-conjugated secondary antibody. For p21, MDMX and actin detection, membranes were probed with a rabbit polyclonal anti-p21 (C-19), anti-MDMX (A300), and anti-actin (C11), respectively, followed by an anti-rabbit horseradish-peroxidase (HRP)-conjugated secondary antibody. For loading control, membranes were stripped and reprobed with a mouse monoclonal anti-yeast phosphoglycerate kinase (Pgk1p) or anti-GAPDH (6C5). For analyses of mitochondrial and cytosolic fractions, membranes were reprobed with the loading controls mouse monoclonal anti-GAPDH (6C5) or anti-Cox4 (F-8), used to exclude putative contamination of cytosolic and mitochondrial fractions, respectively. All antibodies were purchased from Santa Cruz Biotechnology (Frilabo, Porto, Portugal), except the anti-MDMX from Bethyl Laboratories (bioNova cientifica, Madrid, Spain) and the anti-Pgk1p from Alfagene (Molecular probes, Carcavelos, Portugal). The signal was detected with the ECL Amersham kit from GE Healthcare (VWR, Carnaxide, Portugal) and with the Kodak GBX developer and fixer from Sigma-Aldrich (Sintra, Portugal). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat, Marne La Vallée, France).

2.1.9 Co-immunoprecipitation assay

To perform the co-immunoprecipitation (co-IP), the Pierce Classic Magnetic IP and Co-IP Kit from Thermo scientific (Dagma, Carcavelos, Portugal) was used according to the manufacturer's instructions. Briefly, cells were plated at a final density of 5 × 105 cells/plate and incubated for 24 h. Cells were then treated with 25, 50 and 75 µM of OXAZ-1 or DMSO only for 16 h. After cell lysis and protein lysate separation, 500 µg of total protein was incubated with 2 µL of mouse monoclonal anti-p53 (DO-1) or with mouse immunoglobulin G (IgG, control) from Santa Cruz Biotechnology (Frilabo, Porto, Portugal), overnight at 4 °C. Immunocomplexes were immunoprecipitated using magnetic beads. Detection of p53, MDM2, MDMX, and GAPDH (loading control) in whole cell lysate (input) and in immunoprecipitated fraction was performed by Western blot analysis as in section 2.1.8

2.1.10 Analysis of mitochondrial transmembrane potential (Δψm)

For analysis of $\Delta\psi_m$, HCT116 $p53^{+/+}$ tumour cells were plated in 6-well plates at a final density of 1.5 × 10^5 cells/well. After 24 h incubation, cells were treated with the GI_{50} concentration of OXAZ-1 or DMSO only for 8 h. Cells were harvested and incubated with 1 nM 3,3'-Dihexyloxacarbocyanine Iodide [$DiOC_6(3)$; Alfagene (Molecular probes, Carcavelos, Portugal)] for 30 min at 37 °C; cells treated with 50 µM carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP; Sigma-Aldrich, Sintra, Portugal), for 15 min at 37 ºC, were used as positive control of dissipation of $\Delta\psi_m$ potential.

2.1.11 Analysis of reactive oxygen species (ROS) production

Analysis of intracellular ROS generation was performed by flow cytometry. Briefly, HCT116 $p53^{+/+}$ tumour cells were plated in 6-well plates at a final density of 1.5 × 10^5 cells/well and incubated for 24 h. Cells were then treated with the GI_{50} concentration of OXAZ-1 or DMSO only for 48 h. Cells were harvested and stained with 5 µM CellROX Green Reagent from Life Technologies (Alfagene, Carcavelos, Portugal) for 30 min at 37 ºC.

2.1.12 Flow cytometric data acquisition and analysis

For the flow cytometric analysis, the AccuriTM C6 flow cytometer from BD Biosciences (Enzifarma, Porto, Portugal) and the CellQuest software from BD Biosciences (Enzifarma, Porto, Portugal) were used. For the identification and quantification of cell cycle phases the ModFit LT software (Verity Software House Inc., Topsham, USA) was used.

2.1.13 Computational chemistry

The OXAZ-1 chemical structure was built with MOE and energetically minimized using the MMFF94x force field with a RMS gradient of 0.1. The crystallographic structure of MDM2 protein bound to the MI-63 analog (PDB code: 3LBL) was imported in MOE and the co-crystallized waters were removed. Only the C chain was kept and the residues were protonated to match their state at physiological pH using the protonate 3D module of MOE. The binding pocket was defined by using alpha spheres (sphere that contacts 4 atoms on its boundary and contains no internal atoms) within 5 Å of the MI-63 analog crystallographic pose. For MDMX, a similar procedure was used with the 3LBJ crystallographic structure by removing extra co-crystallized molecules but retaining the WK298 inhibitor located in the p53 experimental docking cleft.

All docking experiments were performed using a triangle matcher placement with a London dG scoring and a MMFF94x force field refinement followed by a GBVI/WSA dG re-scoring (defaults in MOE 2013.08). This protocol was tested by re-docking the co-crystallized inhibitors (MI-63 analog in MDM2 and WK298 in MDMX). The top ranked poses were very similar to the experimental ones and correctly reproduced the main interaction features (as expected, the largest variation was on the terminal carbon chain of WK298).

The results were ranked through their binding affinities and the molecular interactions were interpreted based on the best scored conformation per structure.

2.1.14 Statistical analysis

Data were analysed statistically using the GraphPad software. Differences between means were tested for significance using the Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.2 Results

2.2.1 Identification of OXAZ-1 as potential dual inhibitor of the p53 interaction with MDM2 and MDMX in yeast

In this work, six oxazolopiperidinone lactams (Figure 12) were evaluated as dual inhibitors of the p53 interaction with MDM2 and MDMX using yeast target-directed screening assays. These compounds contain, in the same structure, two privileged scaffolds in MDM2 inhibitors design: the piperidinone and indole skeletons (Sun et al., 2014).

Compounds OXAZ-2, OXAZ-3, OXAZ-4, and OXAZ-5 were synthesised by cyclocondensation reaction of (S)-tryptophanol and the appropriate δ-oxo-esters (Amat et

al., 2007a; Amat et al., 2007b; Pereira et al., 2014). The lactams OXAZ-3, and OXAZ-5 were converted to the *N*-tosylindole derivatives OXAZ-6 and OXAZ-1, respectively, with high yields (Amat et al., 2007b).

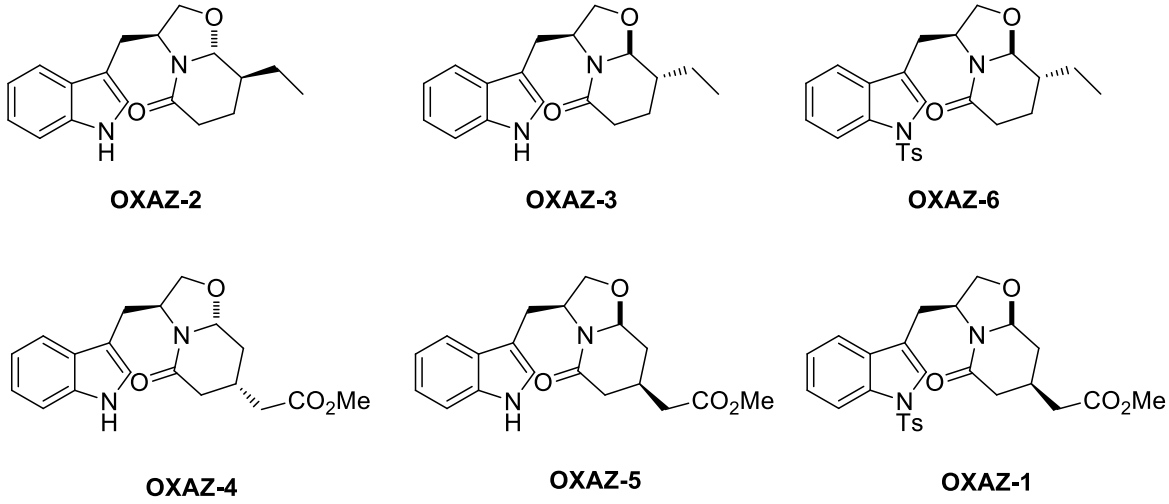

Figure 12. Chemical structure of tryptophanol-derived oxazolopiperidone lactams evaluated in yeast.

The effect of the synthesized compounds as inhibitors of the p53-MDM2/X interaction was thereafter tested using previously developed yeast target-directed screening assays (Leão et al., 2013a; Leão et al., 2013b). These yeast assays are based on the fact that human p53 is also a transcription factor in yeast inducing a marked growth inhibition, which is abolished by human MDM2 and MDMX (Figure 13A). As in mammalian cells, inhibitors of the p53-MDMs interaction reduce the impact of MDMs on p53 activity, thus restoring the p53-induced yeast growth inhibition (Figure 13A). The efficiency of these yeast assays to search for inhibitors of the p53 interaction with MDM2 and MDMX was validated by testing nutlin-3a (a known inhibitor of the p53-MDM2 interaction) (Vassilev et al., 2004) and SJ-172550 (a known inhibitor of the p53-MDMX interaction) (Reed et al., 2010), respectively. As in mammalian cells (Graves et al., 2012), also in yeast nutlin-3a did not interfere with the MDMX inhibitory activity, confirming its activity as MDM2-only inhibitor (Table 2; Figure 13B). On the other hand, SJ-172550 did not interfere with the MDM2 inhibitory activity, only reverting the MDMX inhibitory effect (Table 2; Figure 13B).

Table 2. EC_{50} values obtained for the compounds tested in yeast.

Compounds	EC_{50} (μM)	
	p53-MDM2	p53-MDMX
Nutlin-3a	1.6 ± 3.6	> 50
SJ-172550	> 50	12.4 ± 4.3
OXAZ-1	1.0 ± 2.4	2.4 ± 3.7
OXAZ-2	2.3 ± 3.7	> 50
OXAZ-3	2.0 ± 3.3	> 50
OXAZ-4	2.1 ± 2.4	> 50
OXAZ-5	> 50	> 50
OXAZ-6	3.0 ± 3.3	> 50

Yeast cells co-expressing p53 with MDM2 or MDMX were incubated with 0.1 - 50 μM compounds or DMSO only, for 42 h. The EC_{50} (concentration that caused 50% of the reversion effect) values were determined from concentration-response curves of the reversion of the MDM2 or MDMX inhibitory effects obtained for 0.1 - 50 μM compound (see Figure 12B for OXAZ-1 and positive controls). Data are mean ± S.E.M. of 6 independent experiments.

Using this yeast approach, the effect of 0.1 - 50 μM compounds was evaluated. From the concentration-response curves of the reversion of the MDM2 and MDMX inhibitory effects obtained for each compound, it was shown that, with the exception of OXAZ-5, all the other tested compounds presented similar effects to nutlin-3a, reverting the MDM2 inhibitory effect, and therefore behaved as potential inhibitors of the p53-MDM2 interaction (Table 2). In spite of this, only OXAZ-1 reverted the MDMX inhibitory effect, exhibiting a higher potency than SJ-172550 (Table 2). In fact, contrary to nutlin-3a and SJ-172550, OXAZ-1 reverted the inhibitory effects of both MDMs (Table 2; Figure 13B). Additionally, OXAZ-1 did not interfere with the growth of control yeast and yeast expressing p53, MDM2 or MDMX alone (Figure 13C).

In a recent work, it was shown that expression of human p53 in yeast also increased the expression levels of *ACT1 (*described as a yeast p53 target gene) (Leão et al., 2013b), an effect abolished by MDM2 and MDMX, and re-established by nutlin-3a and SJ-172550, respectively (Leão et al., 2013b). Here, it was shown that, similarly to nutlin-3a (for MDM2) and SJ-172550 (for MDMX), the treatment of yeast cells co-expressing p53 with MDM2 or

MDMX with OXAZ-1 (at 10 µM for MDM2 and 20 µM for MDMX) increased the actin protein levels, when compared to yeast treated with DMSO only (Figure 13D).

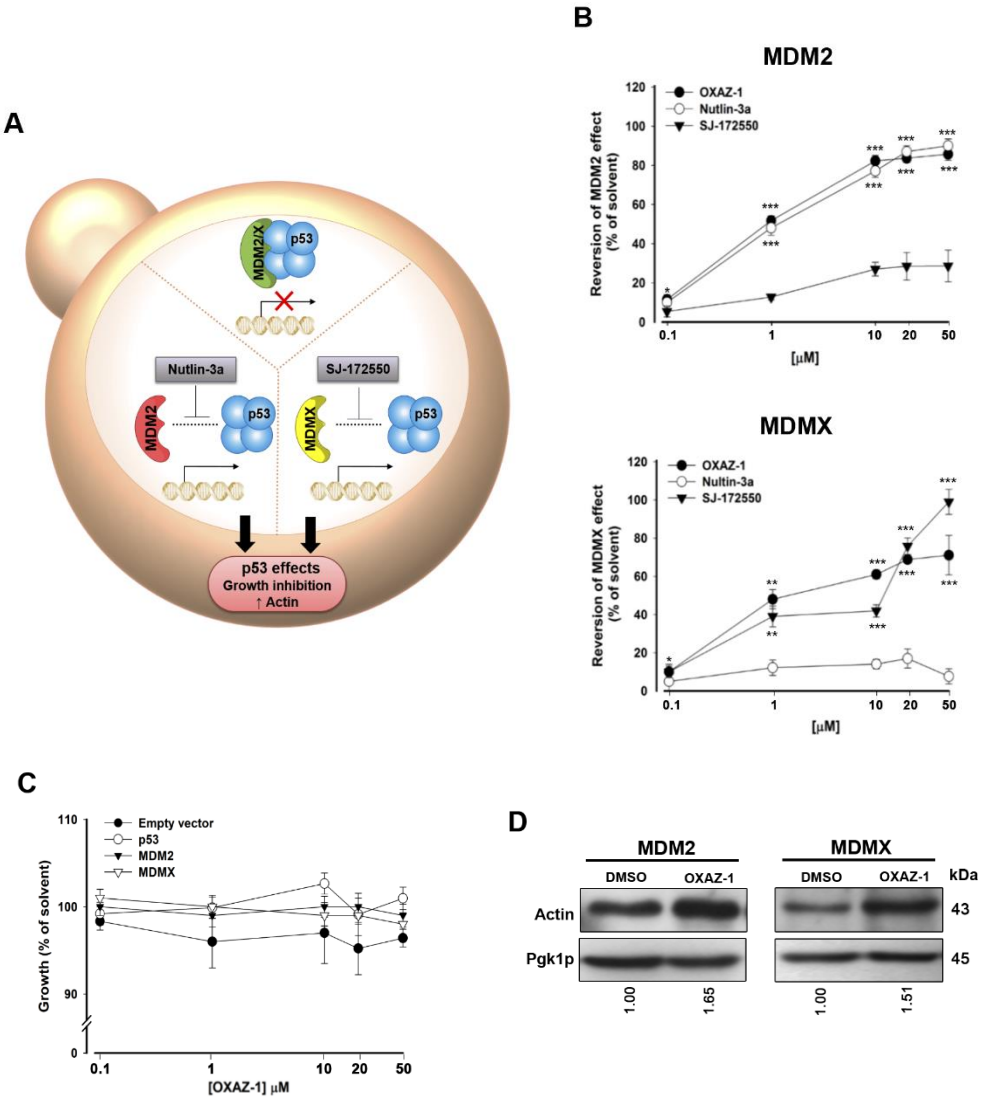

Figure 13. Identification of OXAZ-1 as potential dual inhibitor of the p53 interaction with MDM2 and MDMX, using a yeast screening assay. (A) Schematic representation of the yeast target-directed screening assay. Human MDM2 and MDMX block the growth inhibition and the increase of actin expression levels induced by human wt p53 due to the inhibition of p53 transcriptional activity. Nutlin-3a and SJ-172550 (positive controls) revert the MDM2 and MDMX inhibitory effects, respectively, on p53, re-establishing the p53 activity. **(B)** Effect of 0.1 – 50 µM OXAZ-1, nutlin-3a and SJ-172550 on the reversion of p53-induced yeast growth inhibition by MDM2 and MDMX, after 42 h incubation. The growth of yeast cells co-expressing p53 and MDM2/MDMX was evaluated by CFU counts; results were plotted setting as 100% the growth achieved with yeast cells expressing p53 alone incubated with DMSO only; data are mean ± S.E.M. of six independent experiments; values significantly different from DMSO only are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(C)** Effect of 0.1 – 50 µM OXAZ-1 on the growth of yeast cells expressing p53 alone and control yeast after 42 h incubation. Yeast cell growth was evaluated by CFU counts; results were plotted setting as 100% the growth achieved with DMSO only; data are mean ± S.E.M. of five independent experiments; values are not significantly different from DMSO only ($p > 0.05$). **(D)** Effect of 10 µM OXAZ-1 on the actin protein levels of yeast cells co-expressing p53 and MDM2/MDMX after 42 h incubation. Immunoblots represent one of two independent experiments; Pgk1p was used as loading control.

Together, the results obtained in yeast indicated that *N*-tosylindole OXAZ-1 was a potential dual inhibitor of the p53 interaction with MDM2 and MDMX. With the absence of a direct effect of OXAZ-1 on wt p53 activity, the results obtained may indicate that as inhibitor of p53-MDM2/X interaction, OXAZ-1 may act on MDM2 and MDMX.

2.2.2 OXAZ-1 induces a p53-dependent growth inhibitory effect with inhibition of the p53-MDM2/MDMX interaction in human tumour cells

The tumour cell growth inhibitory potential of OXAZ-1 and the contribution of the p53 pathway to its activity were thereafter ascertained using the human colon adenocarcinoma HCT116 cell lines with wt p53 (HCT116 $p53^{+/+}$) and its p53-null isogenic derivative (HCT116 $p53^{-/-}$). The GI_{50} values obtained for OXAZ-1, after 48 h treatment, of 25 µM in HCT116 $p53^{+/+}$ cells and of 66.3 µM in HCT116 $p53^{-/-}$ cells (Figure 14A) revealed a p53-dependent growth inhibitory effect by OXAZ-1. In fact, a significant reduction of the potency of OXAZ-1 was observed when the p53 pathway was knocked out in HCT116 $p53^{-/-}$ cells.

In HCT116 $p53^{+/+}$ cells, the growth inhibitory effect of OXAZ-1, at the GI_{50} and twice the GI_{50} ($2xGI_{50}$) concentration, was associated to G0/G1-phase cell cycle arrest (Figure 14B) and to late apoptosis (Figure 14C). The activation of a p53-dependent apoptotic pathway by OXAZ-1 was also supported by the occurrence of an increase of cleaved PARP, a product of caspase-3 activation, in $p53^{+/+}$, but not in $p53^{-/-}$, HCT116 cells (Figure 14D).

To further ascertain whether OXAZ-1 had effects on tumour cells consistent with an inhibition of the p53 interaction with MDM2 and MDMX, the protein levels of p53 and of p53 target genes were checked by Western blot analysis in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. The obtained results showed an increase of the p53 baseline levels in HCT116 $p53^{+/+}$ cells upon 25 µM OXAZ-1 treatment (Figure 15A). Moreover, 25 µM OXAZ-1 increased the protein levels of major p53 transcription targets in $p53^{+/+}$, but not in $p53^{-/-}$, HCT116 cells, namely of MDM2, MDMX (recently recognised as a p53 target gene) (Li et al., 2010; Phillips et al., 2010), the cell cycle regulator p21, and the apoptotic proteins of the Bcl-2 family, Puma and Bax (Figure 15A). Moreover, the ability of OXAZ-1 to block the intracellular interaction of p53 with MDM2 and MDMX, in HCT116 p53+/+ cells, was further demonstrated by co-IP (Figure 15B). The obtained results confirmed that OXAZ-1 inhibited the interaction of p53 with both MDM2 and MDMX, particularly at 50 and 75 µM. In fact, at these concentrations of OXAZ-1, little or no MDM2 co-immunoprecipitated with p53, and a visible decrease of the amount of MDMX co-immunoprecipitated with p53 was observed (Figure 15B).

Additionally, in tumour cells with a decreased wt p53 activity by MDM2 and MDMX, OXAZ-1 reduced the MDM2-mediated p53 degradation and the MDM2- and MDMX-inhibitory

effect on p53 transcriptional activity, leading to the subsequent up-regulation of p53 target genes, which supports a potential inhibition of MDM2 and MDMX by OXAZ-1.

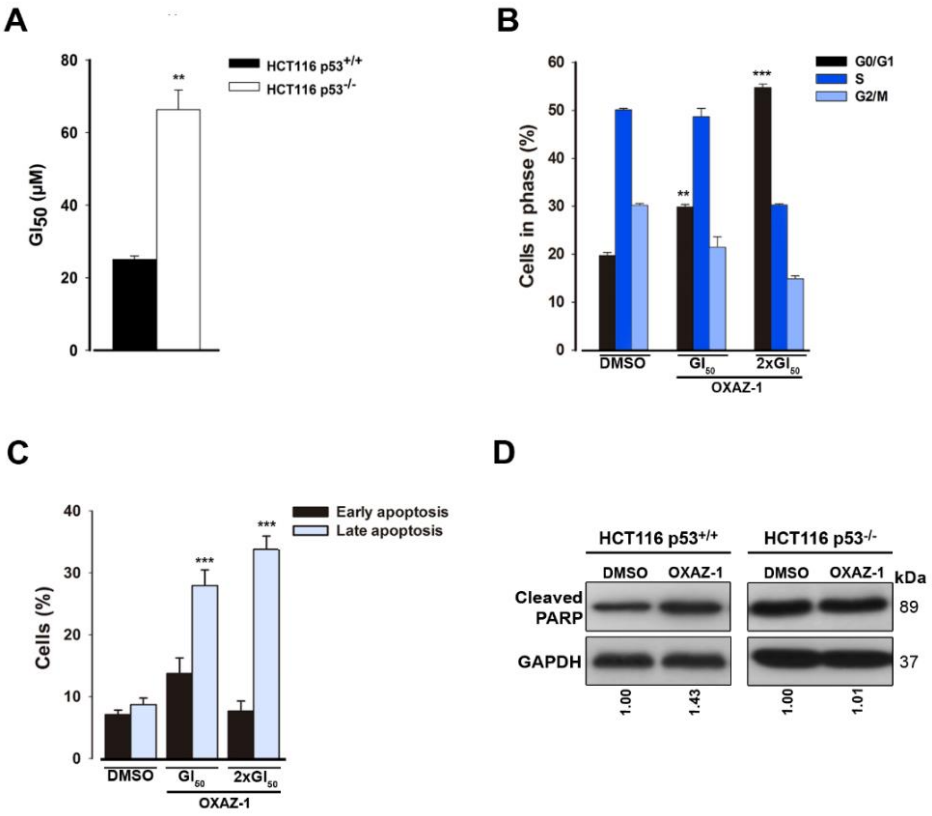

Figure 14. OXAZ-1 induces a p53-dependent growth inhibition associated with cell cycle arrest and apoptosis in human colon adenocarcinoma HCT116 tumor cells. (A) The GI50 concentration of OXAZ-1 was determined in p53+/+ and p53-/- HCT116 cells, after 48 h incubation, using the SRB assay. Data are mean ± S.E.M. of four independent experiments; values significantly different from HCT116 p53+/+ cells are indicated (**p < 0.01). **(B)** OXAZ-1 induces G0/G1-phase cell cycle arrest in HCT116 p53+/+ cells. The effect was determined after 24 h treatment with 25 μM (GI50) and 50 μM (2xGI50) of OXAZ-1; cell cycle phases were analyzed by flow cytometry using PI and quantified using ModFit LT software; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (**p < 0.01, ***p < 0.001). **(C)** OXAZ-1 induces late apoptosis in HCT116 p53+/+ cells. The effect was determined after 24 h treatment with 25 μM (GI50) and 50 μM (2xGI50) of OXAZ-1. Apoptosis was analyzed by flow cytometry using FITC-Annexin V and PI; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (***p < 0.001). **(D)** OXAZ-1 leads to PARP cleavage in p53+/+, but not in p53-/-, HCT116 cells. Western blot analysis was performed after 24 h treatments with 25 μM OXAZ-1 or DMSO only; immunoblots represent one of two independent experiments; GAPDH was used as loading control

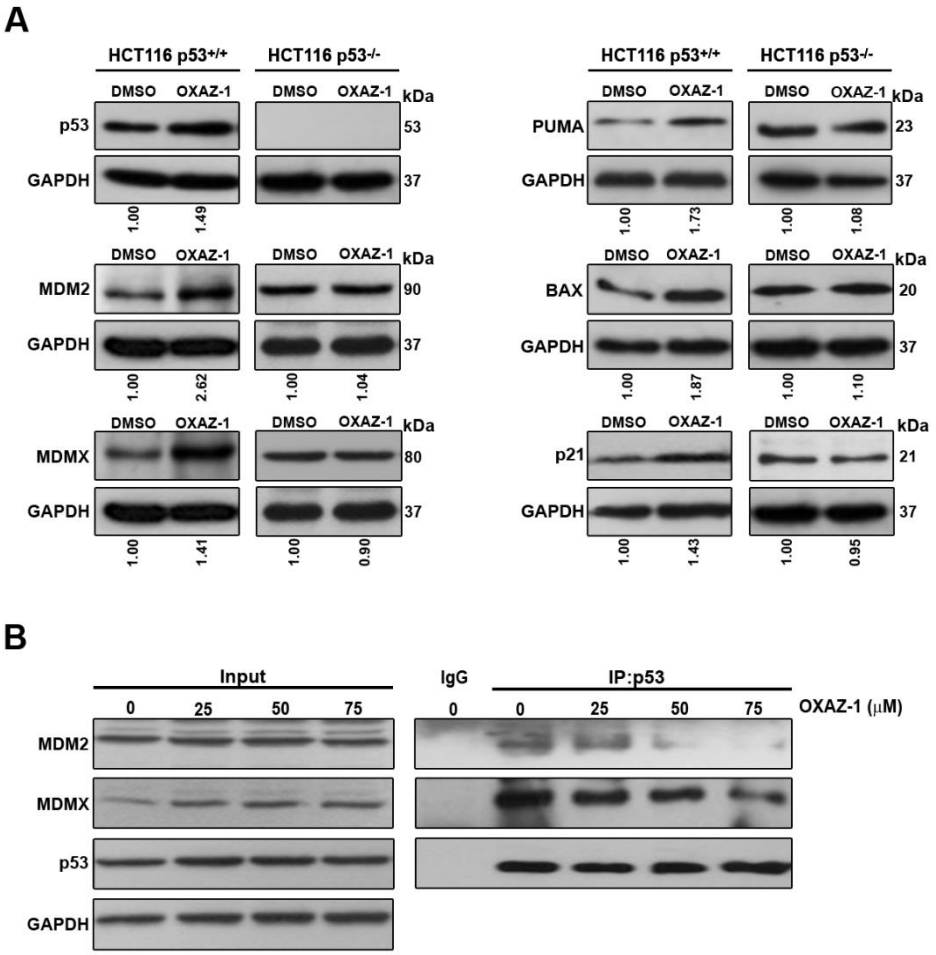

Figure 15. OXAZ-1 leads to p53 stabilization and to the up-regulation of p53 target genes by blocking the p53 interaction with MDM2 and MDMX in HCT116 p53+/+ tumor cells. **(A)** OXAZ-1 increased the protein levels of p53, MDM2, MDMX, p21, Puma and Bax in p53+/+, but not in p53-/-, HCT116 cells. Western blot analysis was performed after 24 h treatment with 25 μM OXAZ-1 or DMSO only; immunoblots represent one of two independent experiments; GAPDH was used as loading control. **(B)** HCT116 p53+/+ cells were treated with 25, 50 and 75 μM OXAZ-1 or DMSO only for 16 h, followed by IP with p53 or mouse immunoglobulin G (IgG) antibodies, and Western blot with MDM2, MDMX and p53 antibodies; whole cell lysate (input); GAPDH was used as loading control.

In addition, in HCT116 $p53^{+/+}$ tumour cells, it was observed that 25 μM OXAZ-1 led to a marked increase of ROS production and $\Delta\psi_m$ dissipation (Figure 14A). Moreover, besides the increase of Bax production (Figure 16A), 25 μM OXAZ-1 triggered the translocation of this mitochondrial protein of the Bcl-2 family from the cytosol to mitochondria (Figure 16B), a typical event of the mitochondrial apoptotic pathway. Finally, an increase of the cyt *c* levels was observed in the cytosolic fraction of tumour cells after 24 h treatment with 25 μM OXAZ-1, indicating its release into the cytosol from mitochondria (Figure 16C). Together, these results showed that OXAZ-1 was a potent inducer of a mitochondrial-dependent apoptotic cell death in human tumour cells.

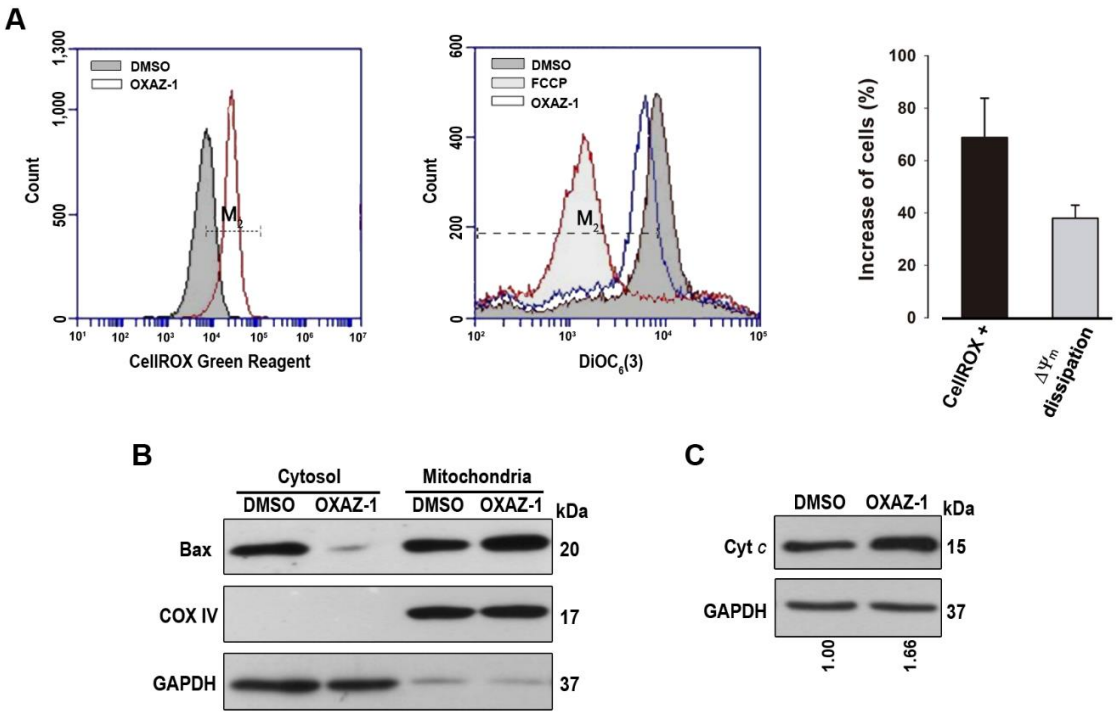

Figure 16. OXAZ-1 induces a mitochondrial-dependent apoptotic pathway in HCT116 p53$^{+/+}$ tumour cells. (**A**) HCT116 p53$^{+/+}$ cells were treated for 8 h (in $\Delta\psi_m$ analysis) or for 48 h (in analysis of ROS production) with 25 μM OXAZ-1 or DMSO only. For assessment of ROS production and $\Delta\psi_m$, cells were stained with CellROX and $DIOC_6(3)$, respectively, and analysed by flow cytometry; histograms represent one of two independent experiments; M2 cursor indicates the subpopulation analysed; values correspond to the increase in the percentage of CellROX positive cells (in analysis of ROS production) and of cells with $\Delta\psi_m$ dissipation obtained after treatment with OXAZ-1 and are mean ± S.E.M. of two independent experiments. In the $\Delta\psi_m$ analysis, FCCP was used as positive control. **(B)** 25 μM OXAZ-1 induces translocation of Bax from cytosol to mitochondria. **(C)** 25 μM OXAZ-1 triggers mitochondrial cyt *c* release into the cytosol after 24 h treatment. Immunoblots represent one of two independent experiments; GAPDH and COX-IV were used as loading controls of cytosolic and mitochondrial fractions, respectively.

2.2.3 MDMX-overexpression tumour cells are highly sensitive to OXAZ-1

Tumour cells overexpressing MDMX have been described as fairly insensitive to MDM2-only inhibitors (e.g. nutlin-3a), due to its inability to inhibit the p53-MDMX interaction, leading to an incomplete restoration of p53 activity (Graves et al., 2012; Patton et al., 2006; Wade et al., 2006). Based on this, the effect of OXAZ-1 was tested in human breast cancer MCF-7 cells, a well-known MDMX-overexpressing tumour cell line (Graves et al., 2012). As expected, a similar growth inhibitory effect to that observed in HCT116 p53$^{+/+}$ cells was achieved with OXAZ-1 in MCF-7 cell lines (GI_{50} value of 23.5 ± 2.5 μM). Additionally, as in HCT116 p53$^{+/+}$ cells, also in MCF-7 tumour cells, the OXAZ-1 growth inhibitory effect was associated to a G0/G1-phase cell cycle arrest (Figure 17A) and to a late apoptotic cell death

(Figure 17B) with PARP cleavage (Figure 17C). Moreover, also in MCF-7 tumour cells, 23.5 μM OXAZ-1 led to the stabilization of p53 protein levels and to the up-regulation of p53 transcription targets involved in cell cycle (p21) and apoptosis (Bax) (Figure 17D).

Altogether, these results showed that also in MDMX-overexpression tumour cells OXAZ-1 was able to activate the p53 pathway predominantly inhibited by MDMX.

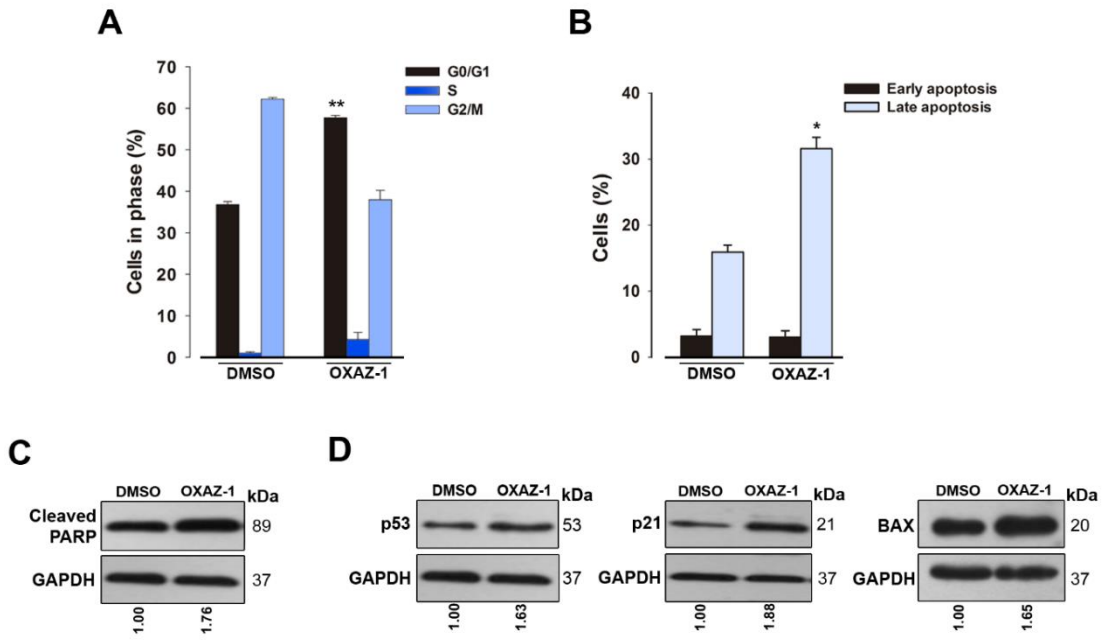

Figure 17. OXAZ-1 exhibits antitumour activity against MDMX-overexpressing human breast adenocarcinoma MCF-7 tumour cells through activation of the p53 pathway. (A) OXAZ-1 induces G0/G1-phase cell cycle arrest in MCF-7 cells. The effect was determined after 24 h treatment with the $2xGI_{50}$ (47 μM) of OXAZ-1; cell cycle phases were analysed by flow cytometry using PI; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (**p < 0.01). **(B)** OXAZ-1 induces late apoptosis in MCF-7 cells. The effect was determined after 24 h treatment with the $2xGI_{50}$ concentration of OXAZ-1. Apoptosis was analysed by flow cytometry using FITC-Annexin V and PI; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (*p < 0.05). **(C, D)** OXAZ-1 leads to PARP cleavage **(C)**, and increases the protein levels of p53, p21 and Bax **(D)** in MCF-7 tumour cells. Western blot analysis was performed after 24 h treatments with 23.5 μM OXAZ-1 or DMSO only; immunoblots represent one of two independent experiments; GAPDH was used as loading control; band intensities were normalized against the control sample (DMSO), which was set as 1.

2.2.4 OXAZ-1 sensitizes tumour cells to chemotherapeutic drugs

It was also investigated if OXAZ-1 increased the sensitivity of tumour cells to the effects of conventional chemotherapeutic drugs such as doxorubicin and etoposide. For that, we

investigated the effect of very low concentrations of OXAZ-1 (approximately the GI_5 to GI_{10} concentration; for which no significant effects on tumour cell growth were observed), on the growth of HCT116 $p53^{+/+}$ cells in combination with increasing concentrations of doxorubicin and etoposide (Figure 18). The results showed that OXAZ-1 significantly increased the sensitivity of tumour cells (from 12% to 19%) at the three concentrations tested of doxorubicin and etoposide.

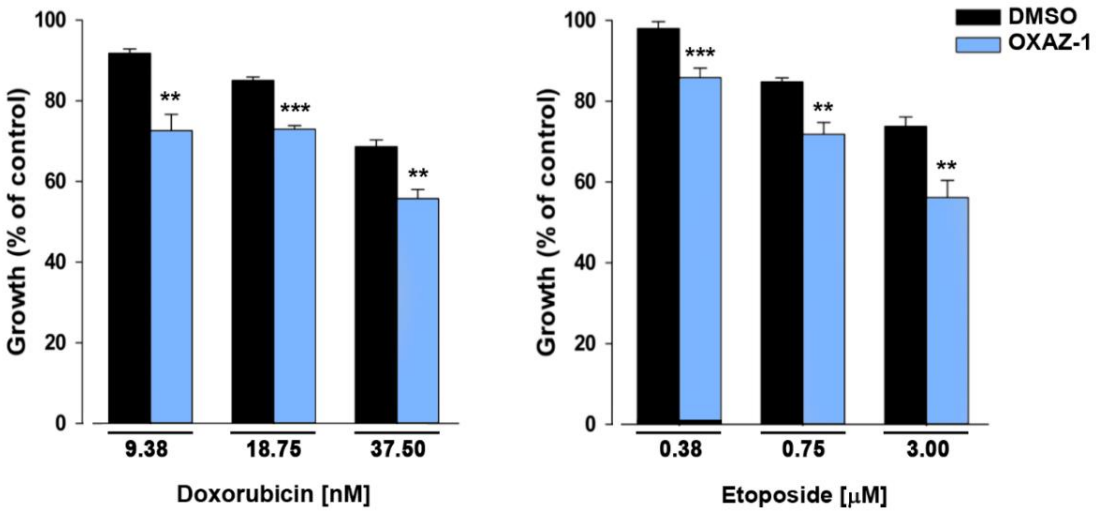

Figure 18. OXAZ-1 sensitizes tumour cells to the effects of doxorubicin and etoposide. The effect of the compounds on cell growth was analysed following 48 h incubation, using the SRB assay. HCT116 $p53^{+/+}$ tumour cells were treated with increasing concentrations of doxorubicin (9.38 to 37.5 nM) or etoposide (0.38 to 3.00 μM) in the presence of a very low concentration (between GI_5 to GI_{10}) of OXAZ-1 or DMSO only. Data are mean ± S.E.M. of three to four independent experiments. Values significantly different from DMSO are indicated (**$p < 0.01$; ***$p < 0.001$).

2.2.5 Analysis of the predicted binding model of OXAZ-1 to MDM2 and MDMX supports that OXAZ-1 binds to both MDM proteins

The OXAZ-1 molecule, presenting the highest percentage of reversion effect on both MDM2 and MDMX, was used in a molecular docking study to shed light on the molecular mode of action. The OXAZ-1 molecule was docked on the MDM2 and MDMX hydrophobic clefts using the Molecular Operating Environment (MOE) software (MOE, version 2013.08) (Chemical et al., 2013) and the crystallographic structures with PDB code 3LBL (MDM2) and 3LBJ (MDMX) allowing ranking the docking poses by binding affinities. On the following discussion, only the top ranked conformation was used.

Recently (Soares et al., 2014b) we have re-docked the MI-63 analog co-crystallized MDM2 ligand present in the 3LBL structure and a similar predicted pose was obtained, with a root

mean square deviation between the heavy atoms of the predicted and co-crystallized poses of 1.65 Å. The WK298 molecule was also re-docked on the MDMX using the 3LBJ structure. It was found that the protocol could also reproduce the crystallographic pose re-creating, this way, the main interactions between the molecule and the protein structure. This defined a rigid protein docking protocol that was also used to predict the pose for OXAZ-1 in MDM2 and MDMX and is described in the experimental section.

MDM2 and MDMX share high sequence homology in their p53 binding domains and interact with three key hydrophobic residues of p53: *Phe19*, *Trp23* and *Leu26*. Between MDM2 and MDMX, p53 interacts in a similar but not identical hydrophobic cleft with the same three *Phe19*, *Trp23* and *Leu26* pockets although for instance, around the latter, some residues are different between both structures (Zhao et al., 2013a).

The docking of OXAZ-1 in MDM2 and MDMX led to a similar prediction for the binding energy: -6.76 kcal/mol in MDM2 and -6.80 kcal/mol in MDMX. However, a visual inspection of the binding interactions established between OXAZ-1 with MDM2 and MDMX shows two different binding modes.

Figure 19 shows the two proteins superposed with the OXAZ-1 top ranked docking poses obtained for each protein. In both poses, the aromatic part of the tosyl moiety is located in the *Trp23* pocket, mimicking this hydrophobic interaction. However, the remaining of the molecule interacts with the two proteins in different zones.

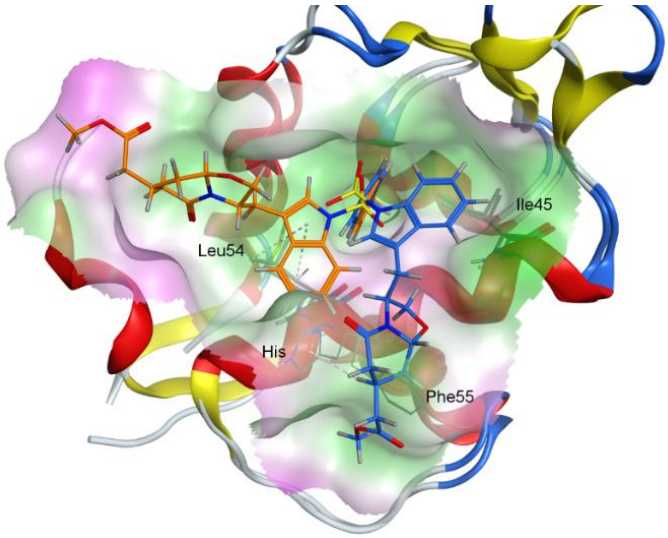

Figure 19. Docking pose of OXAZ-1 within the MDM2 and MDMX hydrophobic clefts limits depicted with a surface (in green, hydrophobic; in pink, hydrophilic areas). *LEU54* and *ILE61* are highlighted and CH-π interactions are displayed through doted lines. The OXAZ-1 pose found with the MDM2 structure is represented with carbon atoms in orange and the one for MDMX is represented with carbon atoms in blue.

In MDM2, the indole moiety of OXAZ-1 makes CH-π interactions with *Leu54* and this moiety occupies the area reserved for hydrophobic interactions by *Leu26* of p53. In MDMX, the MDM2 *Phe55* is substituted by an histidine with a different side chain conformation that decreases the size of the cleft near *Leu54* and prevents a similar pose as in MDM2. However, this histidine is able to make a CH-π interaction with OXAZ-1 allowing the carbon chain of OXAZ-1, containing the piperidone moiety, to sit on a cleft that runs alongside this residue. The indole moiety in this pose is located in the region defined by the p53 *Phe19* residue.

2.3 Discussion

In this work, a yeast target-directed assay was used for the screening of inhibitors of the p53-MDM2/X interaction. From the analysis of a small library of tryptophanol-derived oxazolopiperidone lactams, the *N*-tosylindole OXAZ-1 was identified as a potential dual inhibitor of the p53 interaction with MDM2 and MDMX. The assessment of the predicted binding model of OXAZ-1 to MDM2 and MDMX, by computational docking, and the co-IP assay in tumor cells supported the results from yeast, showing that OXAZ-1 is a putative inhibitor of MDM2 and MDMX, blocking their interactions with p53.

The analysis of the *in vitro* antitumour activity revealed that the tumour growth inhibitory effect of OXAZ-1 was highly dependent on p53. Moreover, in conformity with the results from yeast, OXAZ-1 exhibited typical hallmarks of a dual inhibitor of MDM2 and MDMX (Li and Lozano, 2013). Particularly, it decreased the proliferation of wt p53-carrying tumour cells, an effect associated with the induction of cell cycle arrest and apoptosis. Additionally, it led to the stabilization of the p53 protein levels, that indicates an inhibition of the MDM2-mediated p53 degradation by OXAZ-1, and up-regulation of the p53 transcriptional activity increasing the protein levels of the p53 target genes MDM2, MDMX, p21, Bax and Puma. Finally, it presented reduced antitumour activity against tumours without p53. Beyond that, like RO-5963, OXAZ-1 efficiently reduced the proliferation of wt p53-carrying tumour cells with high levels of MDMX (MCF-7 cells). In fact, also in MDMX-overexpression tumour cells, OXAZ-1 induced cell cycle arrest and apoptosis, led to p53 stabilization, and up-regulated p53 transcription targets involved in cell cycle and apoptosis. OXAZ-1 therefore overcame the commonly reported (Graves et al., 2012; Patton et al., 2006; Wade et al., 2006) resistance of MDMX-overexpression tumour to MDM2-only inhibitors.

Cancer cell mitochondria are structurally and functionally different from their normal counterparts. Additionally, tumour cells are more susceptible to mitochondrial perturbations than the normal cells. Based on this, mitochondrially-targeted agents have emerged as a

promising approach to selectively eradicate chemotherapy-refractory cancer cells (Fulda et al., 2010). In fact, the (re)activation of cell death programmes by pharmacological agents that induce or facilitate mitochondrial membrane permeabilization (MMP) have emerged as an attractive strategy for cancer treatment (Fulda et al., 2010). MMP can be triggered by agents that increase cytosolic calcium or stimulate ROS generation. Moreover, MMP can be favoured by pro-apoptotic proteins of the Bcl-2 family, such as Bax. In fact, during apoptosis, Bax is translocated from the cytosol to mitochondria where it triggers MMP (Chipuk et al., 2004; Fulda et al., 2010). MMP results in the immediate $\Delta\psi_m$ dissipation, with the consequent release of pro-apoptotic factors into the cytosol, such as cyt *c* (Fulda et al., 2010). The results obtained in the present work showed that OXAZ-1 also targets mitochondria of tumour cells. Actually, OXAZ-1 potently triggered a mitochondrion-centered apoptotic cell death characterized by ROS generation, Bax translocation to mitochondria, $\Delta\psi_m$ dissipation, and the subsequent mitochondrial cyt *c* release.

The pharmacological activation of the p53 pathway can be exploited to work in combination with other therapeutic agents to promote cell death via p53-dependent and -independent mechanisms. It is still not completely understood how drug combinations with p53 activators can lead to synergistic enhancement of cell death and improved therapeutic efficacy in the cancer treatment (Hoe et al., 2014). In spite of this, examples were already reported showing the efficacy of such combinations in anticancer treatment. This is the case of nutlin-3a, which has shown excellent results when combined with non-targeted genotoxic agents, such as mitotic inhibitors, CDK inhibitors, DNA-damaging agents, and radiation therapy (Hoe et al., 2014; Wade et al., 2013). Based on this, the potential antitumour activity of OXAZ-1 is therefore strengthened in this work by showing that this compound may prime tumour cells for death induced by chemotherapeutic drugs, such as etoposide and doxorubicin. The prospect of combining conventional chemotherapeutic agents with OXAZ-1 represents a promising strategy to minimize the emergence of resistance and to achieve maximal therapeutic responses with minimal side effects in cancer therapy.

In summary, several evidence are provided in this work for the significant potential of OXAZ-1 as an anticancer agent. The *in vitro* antitumour activity of OXAZ-1 is attributable to the selective activation of the p53 pathway involving the potential dual inhibition of MDM2 and MDMX. Additionally, OXAZ-1 leads to the (re)activation of a mitochondria-mediated cell death programme, being highly effective in combination with conventional therapies by increasing the antitumour outcome at very low concentrations.

Although further work is still required to completely clarify the interaction of N-tosylindole OXAZ-1 with MDM2 and MDMX, relevant insights about its mode of action are provided in this study (Figure 20). Besides, this study opens the way to a new class of selective

activators of the p53 pathway, based on a tryptophanol-derived oxazolopiperidone lactam scaffold, with promising antitumour properties either isolated or in combined therapies. Additionally, the identification of N-tosylindole OXAZ-1 may be the first step toward the development of promising dual inhibitors of the p53-MDM2/MDMX interaction.

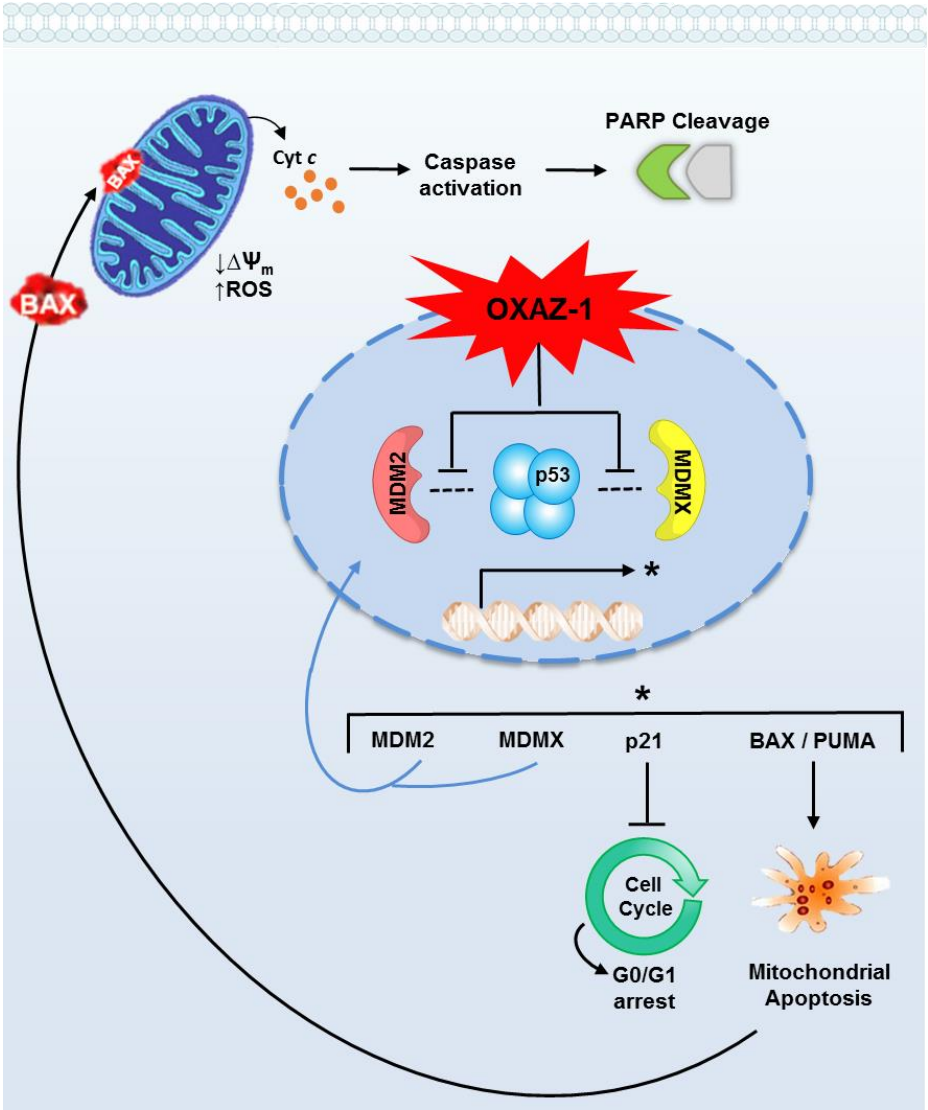

Figure 20. Proposed molecular mechanism underlying OXAZ-1 antitumour activity. As putative dual inhibitor of the p53 interaction with MDM2 and MDMX, OXAZ-1 leads to p53 stabilization and to the subsequent activation of p53 transcriptional activity with increased levels of p53 target proteins, as MDMX, MDM2, p21, Bax and PUMA. The increase of p21 levels leads to a G0/G1 cell cycle arrest. The increase of PUMA and Bax leads to the activation of a mitochondrial apoptotic pathway, involving Bax translocation to mitochondria, ROS production, $\Delta\Psi_m$ dissipation, and cyt *c* release. The release of cyt *c* from mitochondria triggers the activation of a caspase pathway with PARP cleavage; *p53 target genes.

Chapter 3

Development of a yeast target-directed screening assay for biological and pharmacological studies of mutant p53

3 Introduction

About half of all human tumours express inactive mut p53 forms, which often correlates with high resistance to conventional chemotherapy and poor prognosis [reviewed in (Freed-Pastor and Prives, 2012; Girardini et al., 2014)]. Besides their high prevalence, expression of mut p53 is restricted to tumour cells, which makes the pharmacological restoration of wt activity to mut p53 an appealing and selective therapeutic strategy for anti-cancer therapy [reviewed in (Farnebo et al., 2010)].

In the present work, it was developed a new yeast target-directed screening assay for the identification of reactivators of some of the most prevalent mut p53 forms, namely R175H, R273H, R280K and Y220C. In order to validate this yeast assay, known small molecules reactivators of the expressed mut p53 were used. The developed yeast target-directed screening assay is easily adapted for the high-throughput screening of large libraries of small molecules. Additionally, the obtained transformed cells can be used in the study of the biology of the expressed mut p53 forms.

3.1 Material and methods

3.1.1 Plasmids and compounds.

The yeast expression vectors pLS89-(*TRP1*), encoding human wt p53 under the control of *GAL1-10* inducible promoter (kindly provided by Dr. Richard Iggo; Swiss Institute for Experimental Cancer Research, Switzerland), and pTS76-(*LEU2*), encoding human mut p53 R280K, R273H, R175H or Y220C under the control of *ADH1* constitutive promoter (kindly provided by Dr. Gilberto Fronza; IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy), were used.

PRIMA-1 and CP-31398 were purchased from Sigma-Aldrich (Sintra, Portugal), and Phikan083 from Tocris (Biogen Cientifica, Madrid, Spain). All tested compounds were dissolved in DMSO from Sigma–Aldrich (Sintra, Portugal).

3.1.2 Yeast strain, transformation, and growth conditions

For the yeast assay, *S. cerevisiae* (strain CG379, *α ade5 his7-2 leu2-112 trp1-289α ura3-52* [Kil-O]; Yeast Genetic Stock Center) was transformed with a plasmid encoding human wt p53 or the respective empty vector (pLS89; yeast control). To express a panel of human mut p53, yeast cells were transformed with a plasmid encoding the human mut p53 R280K, R273H, R175H or Y220C, or with the respective empty vector (pTS76). The plasmids used

in the yeast transformation process were first amplified in Escherichia coli *DH5α* from Lucigen (Frilabo, Porto, Portugal) and subsequently extracted using the GenEluteTM HP Plasmid Miniprep Kit from Sigma-Aldrich (Sintra, Portugal). After extraction, yeast strains were transformed using the LiAc/SS Carrier DNA/PEG method as described in (Gietz and Schiestl, 2007). For selection of transformed *S. cerevisiae*, cells were routinely grown in a minimal selective medium with 2% (w/w) glucose, 0.7% (w/w) yeast nitrogen base without amino acids from Difco (Quilaban, Sintra, Portugal) and all the amino acids required for yeast growth (50 µg/mL) except leucine (for pTS76 vector) or tryptophan (for pLS89 vector), to approximately 1 OD_{600}.

To induce expression of human proteins, yeast cells were diluted to 0.05 OD_{600} in induction selective medium containing 2% (w/w) galactose and 1% (w/w) raffinose (instead of glucose). Yeast cells were incubated at 30 ºC under continuous orbital shaking (200 r.p.m.) for approximately 30 h (time required by the control yeast, transformed with the empty vector pLS89 or pTS76, to achieve 0.4 OD_{600}). Yeast growth was analysed by counting the number of CFU after 2 days incubation at 30 ºC on Sabouraud Dextrose Agar (Liofilchem).

3.1.3 Yeast protein extraction and Western Blot analysis

To analyse protein expression levels in yeast, samples were lysed with CellyticTM Y Cell Lysis Reagent containing EDTA-free protease inhibitor cocktail from Sigma-Aldrich (Sintra, Portugal). Following protein extraction, whole protein extracts were quantified using the Coomassie staining Bradford from Sigma–Aldrich (Sintra, Portugal). Proteins (40 µg) were electrophoresed on 10% (Acrylamide) SDS-PAGE and transferred to a Whatman nitrocellulose membrane from Protan (VWR, Carnaxide, Portugal). Membranes were blocked with 5% milk and probed with a mouse monoclonal anti-p53 (DO-1), followed by an anti-mouse horseradish-peroxidase (HRP)-conjugated secondary antibody both from Santa Cruz Biotechnology (Frilabo, Porto, Portugal). For loading control, membranes were stripped and reprobed with a mouse monoclonal anti-yeast phosphoglycerate kinase (Pgk1p) antibody from Alfagene (Molecular probes, Carcavelos, Portugal), followed by an anti-mouse horseradish-peroxidase (HRP)-conjugated secondary antibody. The signal was detected with the ECL Amersham kit from GE Healthcare (VWR, Carnaxide, Portugal) and the Kodak GBX developer and fixer are from Sigma-Aldrich (Sintra, Portugal). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat, Marne La Vallée, France).

3.1.4 Yeast growth screening assay

Yeast cells were incubated in induction selective medium in the presence of 0.1 – 200 μM of positive controls (PRIMA-1 and CP-31398 for R280K, R273H and R175H and Phikan083 for Y220C) or 0.1% DMSO only, for approximately 30 h, at 30 ºC, under continuous orbital shaking. Yeast cell growth was analysed as described in section 3.1.2. Results were estimated considering as 1 the growth inhibitory effect caused by the expression of wt p53 in yeast cells incubated with DMSO only.

3.1.5 Statistical analysis

Data were analysed statistically using the GraphPad software. Differences between means were tested for significance using the Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3.2 Results

In this work, the individual expression of human mut p53 R280K, R175H, R273H and Y220C in yeast was confirmed by Western blot analysis (Figure 21A). This analysis showed no significant differences among the protein expression levels of wt and mut p53 forms in yeast. Additionally, the analysis of the growth of yeast cells expressing mut p53 showed that, contrary to wt p53 (Coutinho et al., 2009), none of the human p53 mutants interfered with the yeast cell growth, when compared to the empty vector (Figure 21B).

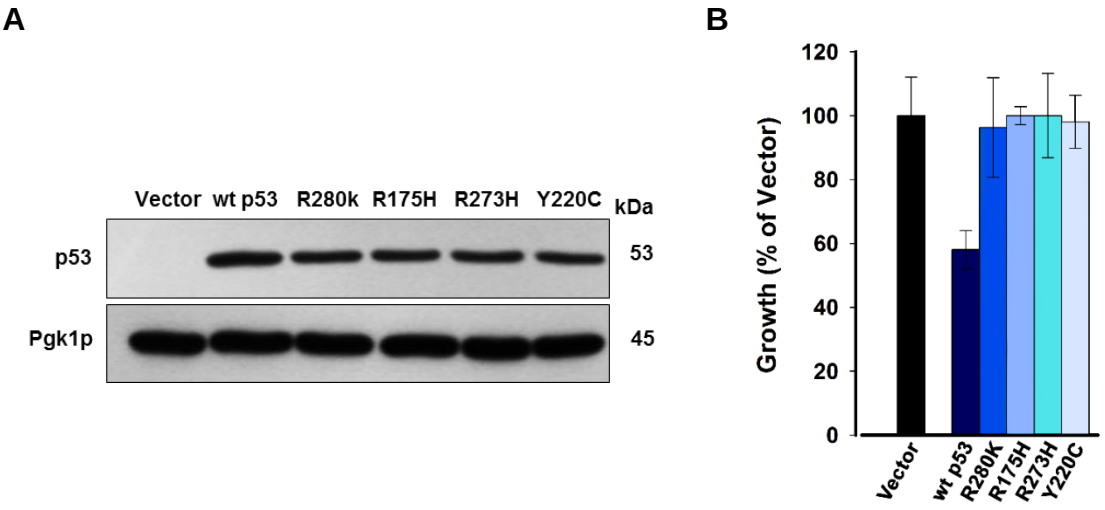

Figure 21. Effect of wt and mut p53 forms in yeast cell growth. (A) Expression of human wt or mut p53 R280K, R175H, R273H or Y220C in yeast was confirmed by Western blot analysis; Pgk1p was used as loading control. **(B)** Effect of mut p53 forms on yeast cell growth; yeast cells were incubated in induction selective medium for 30 h; the growth of yeast expressing mut p53 R280K, R273H, R175H or Y220C was analysed by CFU counts; growth was evaluated considering the growth of control yeast (empty vector) as 100%; data are mean ± S.E.M. of three to five independent experiments. Immunoblots represent one of two independent experiments; Pgk1p was used as loading control;

The efficacy of this yeast assay for the screening of reactivators of mut p53 forms was thereafter assessed by testing known reactivators of the respective mutants, namely PRIMA-1 (Bykov et al., 2002) and CP-31398 (Foster et al., 1999) for human mut p53 R280K, R273H and R175H and Phikan083 for human mut p53 Y220C (Boeckler et al., 2008) (Figure 22). Briefly, this yeast assay is based on the fact that, as in mammalian cells [reviewed in (Freed-Pastor and Prives, 2012)], mut p53 forms are inactive in yeast, and therefore do not induce yeast growth inhibition (Figure 22B). Based on this, reactivators of mut p53 should re-establish the wt p53 activity (inhibitory growth effect) to mut p53 expressed in yeast (Figure 22A).

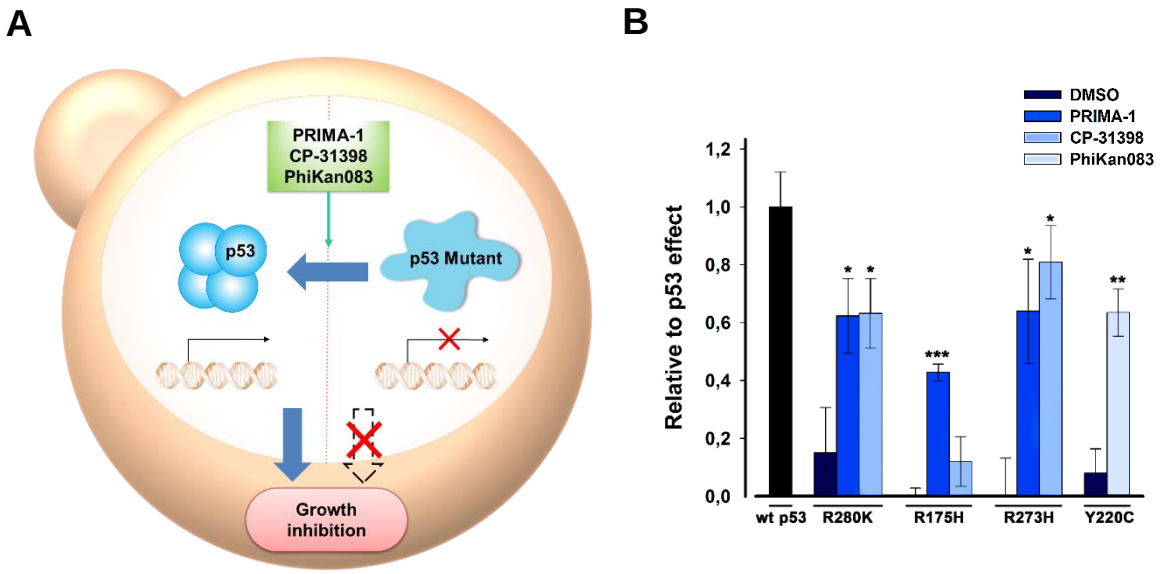

Figure 22. Development of a new yeast target-directed screening assay for the identification of reactivators of mut p53 forms. (*A*) Schematic representation of the yeast target-directed screening assay. Similar to human cells, in yeast, the transcriptional activity of wt p53 is conserved inducing growth inhibition, while the non-functional mut p53 does not affect the yeast growth. In these assay, reactivators of mut p53 re-establish the wt p53 activity (growth inhibitory effect). **(B)** Effect of positive controls on the growth of yeast cells expressing mut p53; yeast cells were incubated in induction selective medium in the presence of 50 μM of PRIMA-1 or CP-31398 (positive controls for R280K, R175H and R273H), or 50 μM of PhiKan083 (positive control for Y220C), or DMSO only, for 30 h; the effect of positive controls on the growth of yeast expressing mut p53 (R280K, R273H, R175H or Y220C) was analysed by CFU counts; the growth inhibitory effect caused by wt p53 expressed in yeast cells incubated with DMSO only was set as 1; data are mean ± S.E.M. of three to five independent experiments; values significantly different from DMSO only are indicated (*$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ unpaired Student's *t*-test).

In this assay, yeast cells expressing R280K, R175H, R273H or Y220C were incubated with 0.1 µM - 200 µM of CP-31398, PRIMA-1 or PhiKan083. The CP-31398 only re-established the wt p53 activity to R280K and R273H at 50 µM, having no significant effects on p53 R175H (Figure 22B). Additionally, our yeast assay was able to detect the reestablishment of the wt p53 function to mut p53 R280K, R175H or R273H by 50 µM of PRIMA-1 (Figure 22B). Finally, the compound PhiKan083 re-established the wt p53-induced growth inhibition to mut Y220C at 50 µM (Figure 22B). It was shown that concentrations of CP-31398, PRIMA-1 and PhiKan083 higher than 50 µM did not increase the p53 activity. Furthermore, at 100 µM and 200 µM, CP-31398 and PRIMA-1, respectively, exhibited cytotoxic effects on control yeast (empty vector). For Phikan083 no cytotoxic effects were observed for all the concentrations tested (data not shown).

3.3 Discussion

The possibility of a more direct and simplified analysis of the human p53 pathway may eliminate the effects of redundant processes on the measured output [reviewed in (Pereira et al., 2012a)]. In fact, the yeast model has already proven its value in the study of human mut p53. Actually, several groups have devoted much time in the study of the transactivation function of *TP53* mutants in yeast, establishing that, when compared to wt p53, nearly all mutants at p53 "*hotspot*" residues lost or retain only a very weak transactivation function [reviewed in (Bisio et al., 2014)]. For example, yeast p53 transactivation assays have been extensively used to study the impact of mutations on wt p53 transcriptional activity (Andreotti et al., 2011; Monti et al., 2007; Monti et al., 2011). Additionally, the impact of using "humanized yeast systems" in the identification of pharmacological regulators of human p53 has already been proven by our group (Leão et al., 2013a; Leao et al., 2013; Leão et al., 2013b).

The selection of the mut p53 forms to be expressed in yeast relied on its prevalence in human tumours, and on its association to resistance to conventional chemotherapeutic agents. In addition, it was also our intention to express both structural (R175H and Y220C) and contact (R273H and R280K) mut p53s (Boeckler et al., 2008; Cho et al., 1994). Among the mut p53 tested, the mut R175H, R273H and Y220C, are some of the most frequent mutations in human cancers [reviewed in (Girardini et al., 2014)]. Moreover, the mut p53 R280K is associate to triple-negative breast cancers (as breast adenocarcinoma MDA-MB-231 tumour cells), which do not respond to the conventional chemotherapy (Foulkes et al., 2010).

This work confirmed the results obtained in *in vitro* and in *in vivo* studies with CP-31398, in which it was shown that this compound was able to re-established the wt p53 activity to mut p53 R280K, R175H and R273H (Foster et al., 1999). In spite of this, contrary to that reported in mammalian cells (Foster et al., 1999; Mehta et al., 2007), in the yeast cell system, Tanner and colleagues were unable to detect reactivation of mut p53 R175H and R273H (Tanner and Barberis, 2004) by CP-31398. Accordingly, in our yeast assay, CP-31398 was also unable to reactivate the wt p53 activity in mut p53 R175H. Further studies must be done in order to confirm this absence of effect of CP-31398 on mut p53 R175H in yeast.

Concerning to PRIMA-1, *in vitro* studies in human tumour cells showed that this compound restores the sequence specific DNA-binding and transcriptional transactivation to mut p53 R280K, R273H and R175H (Bykov et al., 2002; Bykov et al., 2005; Mehta et al., 2007). Besides, *in vivo* studies reported that PRIMA-1 suppressed the tumour-cell growth in mice by inducing apoptosis (Lambert et al., 2009). Although previous efforts of other authors to demonstrate the activity of this compound as reactivator of mut p53 in yeast were unsuccessful (Andreotti et al., 2011), in this work, it was shown that PRIMA-1 was also able to re-establish the wt p53 function to mut p53 R280K, R175H and R273H in yeast.

Concerning to PhiKan083, identified through a rational design approach, this small molecule selectively binds to a cavity on p53 surface created by the Y220C mutation, restoring the wt p53 activity to mut p53 Y220C in human tumour cells (Liu et al., 2013). Our yeast assay was also able to reproduce these results.

Altogether, in the present work, with the reconstitution of human mut p53 R280K, R175H, R273H and Y220C regulatory pathways in yeast, a simplified yeast target-directed growth inhibitory assay was developed to search for reactivators of some of the most prevalent mut p53 forms. This yeast phenotypic assay is based on simple measurements of the yeast cell growth and therefore can be adapted to the high-throughput screening of large chemical libraries in a cost-effective manner. This cell model can also help in the elucidation of the biology of mut p53 forms.

Chapter 4

General conclusions and final remarks

4 General conclusions and final remarks

Cancer is one of the greatest medical concerns of the current aging population. The efficacy of the therapeutic options currently available remain limited and, in most cases, the treatment is accompanied by severe side effects. Accelerating the discovery process of more efficient and selective anticancer agents is therefore of enormous interest, both from economic and medical standpoints [reviewed in (Kumar et al., 2009)].

The p53 protein is a major transcription factor with a crucial role in the regulation of major cellular processes including DNA repair, cell proliferation and death. Its relevance as a tumour suppressor is highlighted by the fact that the p53 activity is lost in all human tumours either by mutation of the *TP53* gene or by inactivation through interaction with negative regulators, particularly MDM2 and MDMX. Based on this, p53 has been considered a key therapeutic target in cancer treatment. In fact, since the discovery of p53 as the “guardian of the genome”, amazing efforts have been developed in order to find molecular strategies to re-establish the p53 function. In this context, inhibitors of the p53 interaction with MDM2 and MDMX and reactivators of mut p53 activity have been considered promising therapeutic approaches in cancers with wt and mut p53 forms, respectively. In spite of this, to date, only two inhibitors of the p53 interaction with MDM2 and MDMX were described. These type of small-molecules are very required for a full activation of the p53 function in tumours with wt p53. Additionally, although several small-molecules have been identified that reactivate the wt p53 function to a wide range of mut p53 forms, only few compounds have been described as true reactivators of mut p53 (Collavin et al., 2010). Additionally, low selectivity to the p53 pathway, unfavorable pharmacokinetic and toxicity profiles make the search for new pharmacological alternatives highly required.

In the present work, using a previously developed yeast-based screening approach, a potential dual inhibitor of the p53 interaction with MDM2 and MDMX was identified, the N-tosylindole OXAZ-1. OXAZ-1 exhibits a p53-dependent *in vitro* antitumour activity against tumour cells retaining wt p53 and distinct levels of MDM2 and MDMX. The antitumour activity of OXAZ-1 is reinforced by its ability to trigger a mitochondria-mediated apoptotic cell death, and by its synergic effects with conventional chemotherapeutic drugs. Although much work remains to be done to completely clarify the molecular mechanism of OXAZ-1, several relevant insights about the mode of action of this compound are already provided in this study. Additionally, this work opens the way to a new class of activators of the p53 pathway (dual inhibitors of MDM2 and MDMX), based on a tryptophanol-derived oxazolopiperidone lactam scaffold, with promising antitumour properties either isolated or in combined therapies.

Moreover, in this work, new yeast target-directed screening assays were developed to search for reactivators of some of the most prevalent human mut p53 forms, namely R280K, R175H, R273H and Y220C. In fact, using this approach, a new reactivator of mut p53 R280K, the oxazoloisoindolinone SLMP53-1, under international patent request (Soares et al., 2014a), was recently discovered by our group. The antitumour activity and molecular mechanism of action of SLMP53-1 was thereafter validated in *in vitro* and *in vivo* animal models. A proof-of-concept was therefore provided for the efficacy of these assays to search for reactivators of mut p53. The enlargement of these assays to other mut p53s will certainly accelerate the discovery of reactivators of these proteins. Finally, it must be highlighted that these yeast assays may be also used to study the biology of these mut p53-R280K, R175H, R273H and Y220C. Actually, these assays may represent a relevant tool for the further elucidation of the mut p53 network, which remains largely unknown.

As a whole, with this project a relevant contribution was provided to the pharmacology of p53 with the identification of a promising small-molecule that can be further explored as potential anticancer agent. Additionally, it improved our anticancer drug discovery strategy with the implementation of new screening assays for relevant target proteins in cancer.

Chapter 5

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5 Bibliography

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