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Targeting p53 in anticancer therapy: searching for new small molecule activators

Beatriz Sampaio da Silva

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Beatriz Sampaio da Silva

TARGETING P53 IN ANTICANCER THERAPY: SEARCHING FOR NEW SMALL MOLECULE ACTIVATORS

Dissertação de Candidatura ao grau de Mestre em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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E um homem não conhece a sua verdadeira ambição até passar por uma tragédia forte, uma tragédia individual. Só se sabe olhar, depois de se aprender.

Gonçalo M. Tavares

Preface

This work was performed at the Laboratory of Microbiology of Biological Sciences Department of Faculty of Pharmacy of University of Porto, in collaboration with Laboratory of Pharmacology and Experimental Therapeutics, IBILI, of Faculty of Medicine of University of Coimbra.

Abstract

Cancer is one of the most concerning diseases of the XXI century, with high mortality rates, mostly due to later diagnosis that restricts surgical options and severe toxic side effects induced by chemotherapy or radiation therapy. The emergence of precision medicine in cancer has changed the therapeutic perspectives, ensuring less toxic side effects. However, the development of resistance to these targeted therapies has led to the search for additional therapeutic strategies namely by their combination with conventional chemotherapeutic agents.

The p53 protein has a major role in tumour development and progression. In fact, p53 is also named the "guardian of the genome" because of its essential role in maintaining genomic integrity and tumour prevention, through regulation of several biological processes, including apoptosis, cell cycle, senescence, and DNA repair. The *TP53* gene is mutated in approximately 50% of all human tumours, which are commonly characterized by poor prognosis. These mutations can lead to an abnormal protein structure with the loss of its sequence-specific DNA binding and subsequent transcriptional activity. Therefore, the restoration of p53 activity might suppress or even reverse tumorigenesis, representing a valuable anticancer therapeutic strategy. Promising therapeutic approaches have been focused on the identification of small molecules capable of activate p53 by restoring the wild-type conformation to mutant p53 forms.

In previous work, our group discovered the small molecule MANIO as a potential activator of wild-type p53 and reactivator of mutant p53 forms, using a yeast-based assay developed by our group. With the present thesis, it was intended to validate the molecular mechanism of action and to study the antitumour activity of MANIO.

Hence, the molecular mechanism of action of MANIO was validated *in vitro* and *in vivo*, demonstrating encouraging antitumor activity. In fact, MANIO presents a p53-dependent tumour growth inhibitory activity associated with cell cycle arrest, apoptosis and modulation of p53 transcriptional targets. Additionally, MANIO has anti-migratory properties and the ability to reactivate high prevalent mutant p53 forms. The non-genotoxicity of the compound *in vitro* was associated with no apparent toxic side effects in *in vivo* mice models. Additionally, it was observed a potent *in vivo* antitumour activity using xenograft mice models of human tumour cells expressing wild-type p53. Collectively, these results support the potential of MANIO as anticancer agent and starting point for the development of improved reactivators of mutant p53.

In conclusion, this thesis contributed to the identification of a new potential anticancer drug candidate with the ability to reactivate mutant p53. MANIO may represent a valuable contribution in the advance of cancer therapies.

Key words: cancer, p53, anticancer agent, targeted therapy

Resumo

O cancro é uma das doenças mais preocupantes do século XXI, com uma alta taxa de mortalidade, maioritariamente devido a um diagnóstico tardio que restringe as opções cirúrgicas, e devido a efeitos secundários severos induzidos tanto pela quimioterapia como pela radioterapia. O aparecimento da medicina de precisão no cancro tem mudado as perspetivas terapêuticas, garantindo menos efeitos tóxicos. Contudo, o desenvolvimento de resistência a estas terapias dirigidas tem levado à procura de novas estratégias terapêuticas, nomeadamente pela combinação com agentes quimioterápicos convencionais.

A proteína p53 tem um importante papel no desenvolvimento e progressão tumoral. De facto, a p53 é também designada por "o guardião do genoma" pelo seu papel na manutenção da integridade genómica e prevenção tumoral, através da regulação de diversos processos biológicos, incluindo ciclo celular, apoptose, senescência, e reparação de DNA. O gene *TP53* está mutado em aproximadamente 50% de todos os tumores humanos, o que é usualmente caracterizado por prognóstico desfavorável. Estas mutações podem levar uma estrutura proteica alterada com perda da capacidade de se ligar a uma sequência especifica de DNA e subsequentemente à perda da sua atividade transcricional. Assim, o restabelecimento da atividade da p53 pode suprimir ou até reverter a tumorigénese, representando uma estratégia terapêutica anticancerígena exequível. As abordagens terapêuticas mais promissoras têm focado a identificação de pequenas moléculas capazes de ativar a p53 pelo restabelecimento da conformação nativa de uma forma mutada da p53.

Num trabalho anterior, o nosso grupo descobriu a pequena molécula MANIO como um potencial ativador da forma nativa e reativador das formas mutadas da p53, usando o modelo de levedura desenvolvido pelo grupo. Com a presente tese, pretende-se validar o mecanismo molecular de ação e estudar a atividade anti-tumoral do MANIO.

Assim, o mecanismo molecular de ação do MANIO foi validade *in vitro* e *in vivo*, demonstrando uma atividade anti-tumoral encorajadora. De facto, o MANIO apresenta uma atividade inibitória do crescimento tumoral dependente da p53, associada à paragem de ciclo celular, apoptose, e à modulação dos alvos transcricionais da p53. Adicionalmente, o MANIO tem propriedades anti-migratórias e a capacidade de reativar formas mutadas da p53 muito prevalentes. A ausência de genotoxicidade do composto *in vitro* foi associada com a inexistência de efeitos tóxicos aparentes em modelos *in vivo* de ratinhos. Para além disso, foi observada uma atividade anti-tumoral *in vivo* potente em

células tumorais humanas que expressavam a p53 nativa. Coletivamente, estes resultados suportam o potencial do MANIO como um agente anticancerígeno e um ponto de partida para o desenvolvimento de melhores reativadores da p53 mutada.

Concluindo, esta tese contribuiu para a identificação de um novo candidato a fármaco anticancerígeno com capacidade de reativação da p53 mutada. MANIO pode representar uma valiosa contribuição para o avanço das terapias contra o cancro.

Palavras chave: cancro, p53, agente anticancerígeno, terapia dirigida

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Abbreviations

A

Apaf-1 (Apoptotic protease-activating factor 1)

ARF (Alternative Reading Frame)

ATM (Ataxia Telangiectasia Mutated)

ATR (Ataxia Telangiectasia and Rad3 related)

B

Bak (Bcl2-antagonist/killer-1)

Bax (Bcl2-associated X)

Bcl-2 (B-cell lymphoma)

BH (Bcl-2 homology)

Bid (BH3 interacting-domain death agonist)

C

CAK (CDK-activating kinase)

Cdc25C (Cell division cycle 25C)

Cdk (Cyclin-dependent kinase)

CdkI (Cyclin-dependent kinase inhibitor)

Chk1 (Cell Cycle Checkpoint Kinase-1)

Chk2 (Cell Cycle Checkpoint Kinase-2)

Cyt *c* (Cytochrome *C*)

D

DBD (DNA Binding Domain)

DISC (Death-Inducing Signalling Complex)

DMSO (Dimethyl Sulfoxide)

DNA (Deoxyribonucleic acid)

DNE (Dominant Negative Effect)

E

EMA (European Medicines Agency)

ETOP (Etoposide)

F

FADD (Fas-Associated Protein with Death Domain)

Fas (First apoptosis signal receptor)

FASAY (Functional Analysis of Separated Alleles in Yeast)

FBS (Fetal Bovine Serum)

FDA (Food and Drug Administration)

G

GADD45 (Growth arrest and DNA damage protein 45)

GLUT (Glucose Transporter)

GOF (Gain of Function)

H

HK2 (Hexokinase 2)

HPV (Human Papilloma Virus)

I

IAP (Proteins-Inhibitors of caspases)

L

LFS (Li-Fraumeni Syndrome)

LOH (Loss of Heterozygosity)

M

mAb (Monoclonal Antibodies)

MCT1 (Monocarboxylate Transporter 1)

MDM2 (Murine Double Minute 2)

MDMX (Murine Double Minute X)

MOMP (Mitochondrial Outer Membrane Permeabilization)

M-phase (Mitosis phase)

Mutp53 (Mutant p53)

O

OD (Oligomerization Domain)

OXPHOS (Oxidative Phosphorylation)

P

p21 (CDK inhibitor)

PARP (Poly ADP Ribose Polymerase)

PBS (Phosphate-buffered saline)

PCNA (Proliferating Cell Nuclear Antigen)

PET (Positron Emission Tomography)

PGM (Phosphoglycerate mutase)

PI (Propidium iodide)

pRb (Retinoblastoma protein)

PRD (Proline-Rich domain)

PTM (post-translational modifications)

PUMA (p53 Upregulated Modulator of Apoptosis)

R

RE (Response Element)

RNA (Ribonucleic acid)

ROS (Reactive Oxygen Species)

S

S. cerevisiae (*Saccharomyces cerevisiae*)

SCO2 (Synthesis of Cytochrome *C* oxidative 2)

Smac (Second mitochondria-derived activator of caspases)

SRB (Sulforhodamine B)

T

TAD (Transactivating Domain)

TIGAR (*TP53*-induced glycolysis and apoptosis regulator)

TKI (Tyrosine Kinase Inhibitor)

TNF-R (Tumour Necrosis Factor Receptor)

TRADD (TNFR1-associated death domain)

TRAIL (TNF-related apoptosis-inducing ligand)

V

VEGF (Vascular Endothelial Growth Factor)

W

Wtp53 (Wild-type p53)

X

XIAP (X-linked IAP)

Y

Y2H (Yeast Two-Hybrid)

1. Introduction

1.1 Neoplastic disease

1.1.1 Cancer history and epidemiology

For long, evidence of susceptibility to malignancies are documented in the literature, mainly in non-primate fossils. Curiously, the oldest description of cancer in humans was documented in an Egyptian papyrus from 1600-1500 BC, referring a breast tumour. Nevertheless, the cancer cases reported from our ancient population not included the most commonly risk factors identified in modern adult humans. Additionally, the scarcity of cases may be explained by the short lifespan, different diet habits, different environmental conditions, and less frequent medical examinations of our ancient population [reviewed in [1, 2]].

Nowadays, cancer is one of the major public health concerns worldwide, with high mortality and incidence rates increasing each year. According to the World Health Organization, in 2012, there were approximately 14 million new cancer cases and 8 million cancer deaths [3]. In Portugal, in the same year, there were 49 000 new diagnosed cancer patients and 24 000 patients died of cancer [3]. Indeed, the prediction for 2020 expects an increase of cancer incidence and mortality worldwide, with the new cancer cases reaching 17 million and the number of cancer deaths of 10 million patients [3]. Moreover, epidemiological evidence showed that there are diverse risk factors that together increase the likelihood to develop cancer, such as infectious diseases, obesity, tobacco, inherited genetic mutations, and immunosuppressive conditions [reviewed in [4]].

1.1.2 Tumourigenesis, a multistage process

The neoplastic disease arises from a multistage process called tumourigenesis, which consists in a sequence of genetic and epigenetic alterations in normal cells. The accumulation of genomic injuries leads to the activation of oncogenes and the inactivation of tumour suppressor genes, altering the cell division control. This uncontrolled cell proliferation promotes the formation of cells with tumourigenic phenotypes [reviewed in [5, 6]]. In this process, different tumour cell phenotypes grow to form a mass, known as tumour, leading to the formation of a heterogeneous tumour [reviewed in [7]].

During the past decades, several characteristics that are required for tumour cells proliferation, survival, and dissemination were identified, being currently known as hallmarks of cancer (Figure 1). In 2000, the first six essential characteristics were proposed, including evading growth suppressors, inducing angiogenesis, resisting cell death, enabling replicative immortality, sustaining proliferative signalling, and activation of

invasion and metastasis. In 2011, two additional emerging hallmarks were suggested, the first one is the reprogramming of cellular metabolism, allowing tumour cells to produce glucose through glycolysis even in the presence of oxygen. The second one is the ability of tumour cells to avoid immune destruction, through modulation of immune cells. In addition, two enabling hallmarks were described as imperative in the acquisition of the hallmarks mentioned above. The increasing genomic instability facilitates the acquisition of mutations in oncogenes and tumour suppressor genes, while the tumour-promoting inflammation allows inflammatory cells to release certain substances that accelerate tumour formation. All of these tumour characteristics contribute to aggressive tumour phenotypes with a gain of metastatic characteristics associated with poor prognosis (Figure 1) [reviewed in [8]].

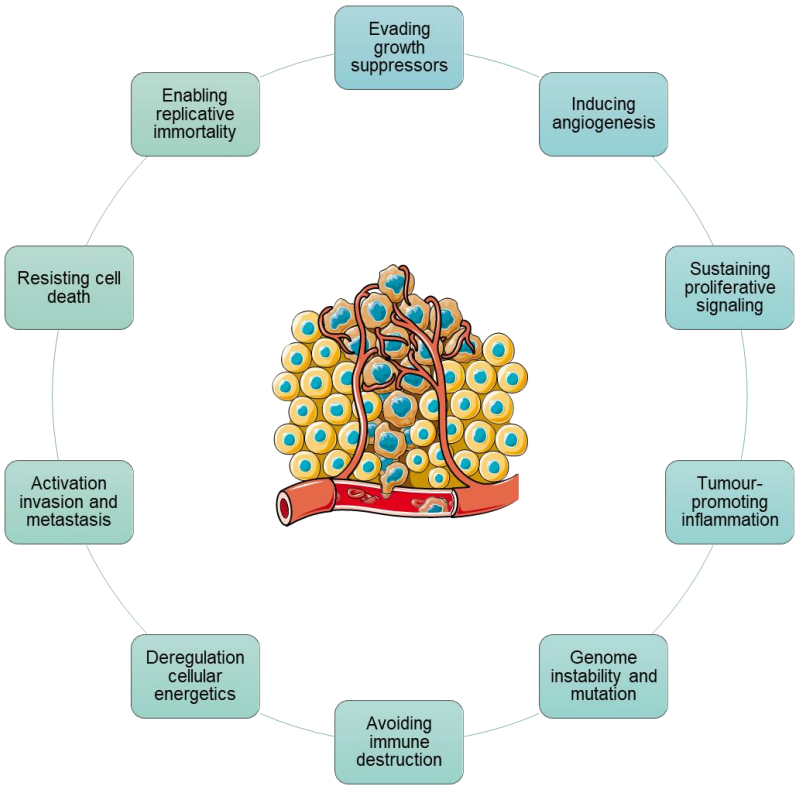

Figure 1 - Hallmarks of cancer. Illustration encompassing the ten required characteristics for tumour cells proliferate, survive, and disseminate.

1.1.3 Cancer therapeutic approaches

Centuries ago, cancer was an incurable disease, even with the use of chemotherapy since the 20th century. In 60's decade, chemotherapy started to be used with a curative intent, in acute lymphoblastic leukaemia and Hodgkin's lymphoma

[reviewed in [9]]. Nowadays, according to the European Society for Medical Oncology (ESMO) guidelines [10-13], the chemotherapy has been administrated in addition to surgery and/or radiation therapy with a curative intent, in the majority of solid tumours.

The conventional chemotherapy can be included in cancer therapy as neoadjuvant and/or adjuvant treatments, corresponding to treatments before or after the surgery, respectively. Neoadjuvant treatments are mainly used to reduce the primary tumour volume, allowing a more efficient resection with negative surgical margins. In the other hand, adjuvant treatments allow the elimination of micrometastases that can possibly exist in the organism without being previously detected, preventing a possible local or distant relapse [reviewed in [14, 15]].

The conventional chemotherapy consists of cytotoxic drugs that target rapidly proliferating cells, like tumour cells, enhancing its sensitivity to DNA damage and triggering cell death. These cytotoxic drugs can be divided into several groups: (1) alkylating agents that form crosslinks between DNA strands, destabilizing DNA during replication; (2) antimetabolites that inhibit the synthesis of DNA/RNA by competing with natural substrates for the active site on an enzyme/receptor; (3) topoisomerase inhibitors, which disrupt the correct unwinding of DNA during replication by interfering with the action of topoisomerase enzymes I/II; (4) microtubular poisons, which block the polymerization or depolymerization of tubulin that inhibits the mitotic spindle; and (5) antitumour antibiotics, which intercalate in the DNA, leading to the overgeneration of reactive oxygen species (ROS) (Table 1) [reviewed in [16-20]].

Table 1 – Cytotoxic drugs for cancer treatment currently used in the clinic.

Drug class	Drugs	Reference
Alkylating agents	Cyclophosphamide, Cisplatin, Ifosfamide, Carboplatin	[17]
Antimetabolites	5-Fluorouracil, Methotrexate, Capecitabine, Gemcitabine	[18]
Topoisomerases inhibitors	Irinotecan, Topotecan, Etoposide, Teniposide	[19]
Microtubular poisons	Paclitaxel, Docetaxel, Cabazitaxel, Vinorelbine	[20]
Antitumour antibiotics	Bleomycin, Doxorubicin, Epirubicin, Mitomycin	[16]

The major limitation of these conventional chemotherapeutics is their non-specificity to tumour cells, also affecting normal cells. This lack of specificity induces several adverse effects in cancer patients, with a negative impact in their quality of life [reviewed in [14, 21]].

Additionally, the selection of conventional chemotherapeutics for treatment is predominantly based on the pathological examination of a tumour. However, even for the same type and stage of cancer, patients may have distinct therapeutic outcomes, since they may have different genetic alterations. Therefore, the concept of precision medicine, i.e. a personalized therapy for each cancer patient depending on its tumour genomic signature, aims to target specific molecules that may be differentially altered in each tumour context [reviewed in [22]]. Therefore, for the inhibition of tumour growth, targeted therapies act on specific protein or genetic aberrations present only or predominantly in tumour cells (Table 2) [reviewed in [14]].

The first targeted therapy used was the endocrine therapy directed to hormone-sensitive tumours, such as breast and prostate carcinomas, which depends on specific steroid hormone receptors to mediate hormone effects on the initiation and progression of neoplastic disease. These endocrine agents are selective modulators that block the hormone receptors (estrogen, progesterone or androgen) or inhibit hormonal synthesis. The most used endocrine agents have been tamoxifen, aromatase inhibitors, and antiandrogens (Table 2) [reviewed in [23, 24]].

Tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAb) are the main categories of the most recent targeted therapies. Tyrosine kinases are involved in the signalling cascade of several biological processes, including growth, differentiation, metabolism, and apoptosis. Therefore, its deregulation plays an important role in tumour cells, leading to abnormal proliferation, anti-apoptosis and angiogenesis. TKIs are small molecules that block specific tyrosine kinases deregulated in tumour cells, such as imatinib, erlotinib and sunitinib (Table 2) [reviewed in [25, 26]]. On the other hand, mAb target tumour cells and can be used to inhibit growth signals and angiogenesis, and to deliver chemotherapy or radiation to tumour cells, minimizing the treatment side effects on healthy cells (Table 2). These mAb are also included in immunotherapy to enhance the immune system through modulation of immune checkpoints (Table 2) [reviewed in [14, 27, 28]].

Another targeted therapy recently approved is based on the synthetic lethality concept, which consists in the loss of cell viability through the synthetic inhibition of one gene when other is already inhibited in tumour cells. PARP (poly ADP ribose polymerase)

inhibitors were the first approved drugs in this therapeutic category and are used in BRCA germline-mutated tumours by inhibiting PARP with subsequent cell death induction (Table 2) [reviewed in [29, 30]].

Table 2 - Targeted drugs for cancer treatment currently used in the clinic.

Drug class	Drug	Target	First approved indication	Approval year	Reference
Endocrine Therapy	Tamoxifen	ER	Breast cancer	1977	[31]
	Letrozole	Aromatase enzyme	Breast cancer	1997	[32]
	Bicalutamide	AR	Prostate cancer	1995	[33]
Tirosine Kinase Inhibitors	Imatinib	BCR-ABL	CML	2001	[25]
	Gefitinib	EGFR	NSCLC	2003	[34]
	Sunitinib	VEGF	Renal cell carcinoma	2006	[35]
	Vemurafenib	BRAF	Melanoma	2011	[36]
Monoclonal Antibodies	Rituximab	CD20	Non-Hodgkin's lymphoma	1997	[37]
	Trastuzumab	Her2	Breast cancer	1998	[38]
	Bevacizumab	VEGF/VEGFR	Coloretal cancer	2004	[39]
	Cetuximab	EFGR	SCC of the Head and Neck	2004	[40]
	Ipilimumab	CTLA-4	Metastatic melanoma	2011	[41]
	Nivolumab	PD-1/PD-L1	Advanced melanoma	2014	[42]
Synthetic lethality	Olaparib	PARP	Advanced ovarian cancer	2014	[43]

ER (Estrogen receptor), AR (Androgen receptor); BCR-ABL (Philadelphia chromosome); EGFR (Epidermal growth factor receptor); VEGF (Vascular endothelial growth factor); BRAF V600E (amino acid substitution at position 600 in BRAF, from valine to a glutamic acid); Her-2 (also known as Erb-B2 receptor tyrosine kinase 2); CTLA-4 (Cytotoxic T-lymphocyte protein 4 precursor); PD-1/PD-L1 (Programmed cell death protein 1/ Programmed cell death ligand 1); PARP (Poly (ADP-ribose) polymerase); CML (Chronic myeloid leukaemia); NSCLC (Non-small cell lung cancer); SCC (Squamous cell carcinoma).

Altogether, targeted therapies allow a more precision therapy than conventional chemotherapy, with less toxic side effects. However, in the past few years, several resistances have been reported associated with these therapies. Therefore, it is still a challenge to treat cancer with high efficiency and low associated undesirable toxicity [reviewed in [44]].

1.2 *TP*53: tumour suppressor gene

Although a high multiplicity of oncogenes and tumour suppressor genes have been implicated in tumourigenesis, the *TP53* is a key tumour suppressor gene with multiple cellular functions. *TP53* encodes the p53 transcription factor protein, which assures the genomic integrity, culminating in a protective effect against tumour development [reviewed in [45]].

The *TP53* gene was discovered in 1979 as an oncogene since its protein bound to the oncogenic T antigen from a simian virus (SV40), inducing a malignant transformation of human and mouse cells [46, 47]. Ten years later, *TP53* was classified as a tumour suppressor gene, since it was ascertained that the discovered of p53 was not the wild-type (wt) form, as considered in the previous study, but instead a mutant (mut) p53 form [48]. In fact, it was the mutp53 form that induced a malignant transformation of cells, while the wt form has a crucial role in suppressing tumour formation through apoptosis, cell cycle arrest, DNA damage repair, senescence, metabolism reprogramming, and inhibition of metastasis and angiogenesis [reviewed in [49, 50]].

The relevance of p53 in cancer greatly increased in the last decades, mainly since the p53 activity is ubiquitously lost in human tumours through two major mechanisms. In one hand, almost 50% of all human tumours harbour a p53 mutation, which attenuates or eliminates its function as tumour suppressor [reviewed in [51, 52]]. Although the remaining tumours retain wtp53, its inactivation is still observed namely due to the amplification or

overexpression of its major negative regulators, the murine double minutes (MDM)2 and/or MDMX [reviewed in [5]].

The activation of p53 is therefore of extreme importance in cancer therapy, considering that the loss of its tumour suppressor function leads to more aggressive tumour phenotypes with subsequent resistance to currently available therapies [reviewed in [53]].

1.2.1 Structure organization of p53 protein

The *TP53* gene is located at 17p13.1 and encoded a homotetramer protein with 393 amino acids and 53 kDa [reviewed in [54, 55]]. p53 is a nuclear phosphoprotein that is capable of transactivation or repression, depending on the presence or not of binding sequences, such response elements (REs) in the promoters [reviewed in [56]].

The p53 protein presents commonly domains of transcription factors, including a *N*-terminal region with a transactivating domain and a proline-rich domain, followed by the central folded DNA-binding domain in the central core of this protein (Figure 2). At its *C*-terminal region, p53 contains an oligomerization domain and a basic regulatory domain (Figure 2) [reviewed in [52]].

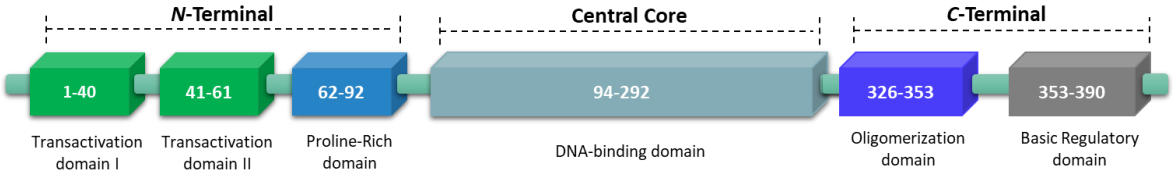

Figure 2 - Schematic representation of human p53 protein structure. p53 protein can be divided in *N*-terminal, central core and *C*-terminal regions. In the *N*-terminal region, p53 contains one transactivation domain (subdivided into two sub domains) and a proline-rich domain. The central core presents the DNA-binding domain, in which most of the missense mutations of p53 occur. In the *C*-terminal region is located an oligomerization domain, responsible for the tetramerization of the protein, and a basic regulatory domain.

The molecular characterization of the p53 functional domains has allowed the identification of interactions with several partners that decode diverse protein functions. The transactivating domain (TAD) interacts with various transcription factors, such as components of the transcription machinery, the transcriptional coactivators p300/CBP (CREB-binding protein) and its negative modulators MDM2/MDMX. The TAD is natively

unfolded and can be divided into two subdomains (TAD1 and TAD2; Figure 2) that can activate transcription independently. Nevertheless, both subdomains present intrinsic disorders, facilitating the binding to several target proteins with high specificity and affinity that allow the TAD to become fully folded [reviewed in [52, 56, 57]]. After the TAD, it is possible to find the proline-rich domain (PRD) linking the TAD to the DNA-binding domain (Figure 2). The PRD is required for apoptosis and growth suppression triggered by p53. This domain is closely related to p53 stability, that is regulated by MDM2, suggesting that the loss of this domain traduces in a higher susceptibility for p53 degradation by MDM2 [reviewed in [56, 57]].

In the central core of p53 protein, it is localized the DNA-binding domain that facilitates the binding of p53 to sequence-specific double-stranded DNA. Additionally, the stability of the full length p53 is mainly dictated by the stability of the DBD, since it melts above the body temperature (44ºC). Moreover, the presence of a zinc ion in this domain is also important for the stability and accurate binding to DNA sequences (Figure 2) [reviewed in [56, 58]].

The *C*-terminal region contains the oligomerization domain (OD) responsible for the formation of a homotetramer based on four monomers, which is necessary for p53 transcriptional activity, through the recognition of p53REs. At the end of this region, the basic regulatory domain is crucial for the negative regulation by post-translational modifications (PTMs), nuclear localization and non-specific DNA binding (Figure 2) [reviewed in [56-58]].

The characteristics and functionality of each domain of p53 have been elucidated in the past years, making possible the understanding of several functions of p53 in normal and tumour cells.

1.2.2 p53 biological functions

The tumour suppressor p53 is considered “the guardian of the genome” since it can regulate the expression of several target genes to normalize a stressed cell state. The two most important cellular responses to stress signals are cell cycle arrest and apoptosis, which are triggered based on damage extension. In fact, in case of limited DNA damage, p53 promotes cell cycle arrest for DNA repair. Nonetheless, in case of severe DNA damage that cannot be repaired, p53 stimulates apoptosis, ensuring the elimination of tumour-prone cells [reviewed in [59, 60]].

Cell cycle regulation by p53

The cell cycle is a common event in the life of a cell. This complex process allows cell growth and proliferation, organismal development, regulation of DNA damage and, when it does not occur, cancer development. The major function of the cell cycle is to replicate meticulously DNA in the chromosomes and therefore divide the copies into two genetically identical daughter cells [reviewed in [61]].

The cell cycle can be subdivided into interphase and mitosis (M) phase. The interphase encompasses two gaps, the G1- and G2-phase, that occur between DNA replication and M-phase. The G1-phase, the first gap of the cell cycle, prepare cells to DNA duplication. This DNA synthesis occurs in S-phase and subsequently G2-phase ensures that cells are prepared to enter in M-phase. M-phase comprises prophase, metaphase, anaphase and telophase that are responsible for the correct segregation of DNA copies into two daughter cells. Although cells are normally dividing, there are other cells that are temporarily or permanently out of the cycle, namely cells in a quiescent state. Thus, these cells are in the G0-phase waiting for external stimulation to enter in G1-phase and start dividing [reviewed in [61, 62]].

Similarly to other cellular processes, the cell cycle is also controlled and regulated to ensure the correct sequence, DNA replication and chromosome separation. Each event of this process is dependent on the correct completion of the earlier events of the cycle, which assure a successful formation of two new cells. This control is made by checkpoints with sensors of damage between phases, preventing the progression of cell cycle until the correct completion of each phase [reviewed in [63]]. There are three key checkpoints with extensive regulation of the cell cycle. The G1/S and G2/M checkpoints are responsible for the verification of DNA damage, before or after S-phase, respectively. These two checkpoints lead to cell cycle arrest to guarantee that cells do not enter in S- or M-phase, respectively, before the repair of DNA damage. Another relevant checkpoint exists in M-phase that leads to the blockage of mitotic progression, if the mitotic spindle is not assembled or if chromosomes are not accurately oriented [reviewed in [62-64]].

The cell cycle regulation is essentially made by complexes containing cyclin-dependent kinases (Cdks) and cyclins. These complexes act in order to phosphorylate specific protein substrates, allowing the progression of each phase of cell division. As indicated by the name, cyclins have a cyclic expression during the cycle, whereas Cdks are always present. Hence, Cdks activation only occurs at a specific time of the cycle with a specific cyclin. Commonly, the cyclin suitable for each phase is synthesized before enters into the respective phase and degraded before the end of the same phase. From

quiescent cells until S-phase, the cyclin D activates Cdk4 and Cdk6, and the cyclin E activates Cdk2. From S- to G2-phase, cyclin A activates Cdk2, which stimulates DNA replication. In G2-phase, cyclin B activates Cdk1 allowing cells to enter into M-phase [reviewed in [62, 64, 65]].

The p53 protein has a key role in the preservation of genome integrity. Therefore, the detection of DNA damage during the cell cycle leads to p53 activation in order to promote the expression of genes involved in cell cycle arrest and DNA damage repair [reviewed in [66]]. In G1/S checkpoint, under unstressed conditions, the increased expression of cyclin D results in the phosphorylation of Cdk4/6, leading to its activation, which in turn allows the phosphorylation of retinoblastoma (pRb) protein, one of the most critical effectors of G1-phase progression. When pRb is hypophosphorylated, it binds to E2F transcription protein, inhibiting transcription. Once Cdks4/6 phosphorylates pRb, it releases E2F protein, allowing the transcription of genes responsible for the S-phase of the cell cycle. pRb remains hyperphosphorylated until mitosis is complete. On the contrary, under a stressed state caused by DNA damage, p53 activates the expression of p21/WAF1, a Cdk inhibitor (CdkI) that inhibits Cdk4/6. Consequently, the inhibition of these kinases blocks the pRb phosphorylation, preventing the transcription of E2F target genes and cell cycle progression (Figure 3). Additionally, p21 binds and inhibits the proliferating cell nuclear antigen (PCNA), which is involved in DNA duplication, thereby avoiding division of damage cells [reviewed in [61, 67, 68]].

Furthermore, as mentioned above, in G2-phase, increased expression of cyclin B complexed with Cdk1 (also known as cdc2) drives the cell through mitosis. For the progression of cells from G2- to M-phase, the Cyclin B/Cdc2 complex needs to be activated by Cell division cycle 25C (Cdc25C) phosphatase, a phosphatase that promotes mitosis, and also by phosphorylation of the complex through Cdk-activating kinase (CAK) [reviewed in [69]]. However, in response to DNA damage or incomplete DNA synthesis, p53 is also capable to participate in the G2-phase arrest of the cell cycle [reviewed in [61]]. Thus, p53 induces G2-phase arrest by modulation of different targets, including p21, Cdc25C, 14-3-3σ protein and Growth Arrest and DNA Damage protein 45 (GADD45) (Figure 3) [reviewed in [67, 69]].

Upon DNA damage in G2-phase, Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3 related (ATR) activate Cell Cycle Checkpoint Kinase (Chk)1 and Chk2 kinases, which phosphorylate Cdc25C [70]. This phosphorylation creates a site for the bind of 14-3-3σ protein, activated at the same time by p53. The 14-3-3σ protein promotes the export of Cdc25C to the cytoplasm, blocking the activation of Cdc2 [71].

Additionally, p53 activates the expression of p21, which directly inhibits Cdc2 [72] and GADD45, which dissociates the two constituents of the complex (Figure 3) [73].

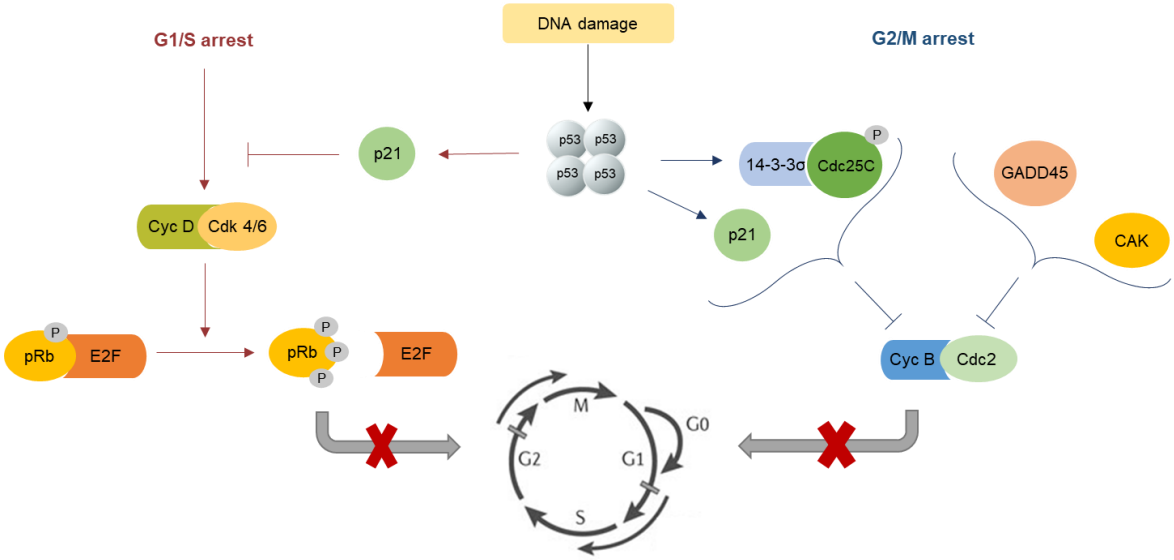

Figure 3 - Regulation of cell cycle by p53 upon DNA damage: induction of G1- or G2-phase cell cycle arrest. In homeostasis conditions, the cell cycle occurs without the interference of p53, from G1-phase to M-phase. In G1/S checkpoint (pink arrows), under unstressed conditions, increased expression of Cyc D promotes Cdk 4 and Cdk 6 phosphorylation and subsequent activation. Typically, pRb is hypophosphorylated, repressing E2F. However, Cdk4/6 activation promotes the phosphorylation of pRb, that leads to a hyperphosphorylated state, which releases E2F from pRb, promoting the progression of the cell cycle. Upon DNA damage, p53 is activated inducing the expression of p21 that inhibits the phosphorylation of Cdk4/6. Under these conditions, E2F is repressed by pRb, preventing the transcription of E2F target genes and cell cycle progression. In G2-phase (blue arrows), another Cyc/Cdk complex must be activated. In this case, for the activation of Cyc B/Cdc2 complex, Cdc25C phosphatase needs to be activated. However, under DNA damage, Cdc25C is phosphorylated by Chk1/Chk2 kinases, creating a site for 14-3-3σ binding. 14-3-3σ is activated by p53, leading to the export of Cdc25C to the cytoplasm, preventing Cdc2 activation. Also, in G2 arrest, p53 induces the expression of p21 that also inhibits Cdc2. Two other proteins also block Cdc2 activation, namely GADD45 and CAK; Cyc: Cyclin; Cdk: cyclin-dependent kinase; pRb: Retinoblastoma protein; P: phosphorylation; Cdc25C: Cell division cycle 25C; GADD45: Growth Arrest and DNA Damage protein 45; CAK: Cdk-Activating Kinase.

Despite the participation of p53 in G1/S and G2/M checkpoints, only G1/S checkpoint is totally p53-dependent. A previous study demonstrated that cells with DNA damage, in which p53 is deleted or mutated, do not exhibit arrest in G1/S checkpoint. However, it was possible to verify the arrest in G2/M checkpoint, which proves the dependence of p53 in G1/S arrest comparatively to G2/M arrest [[74]; reviewed in [68]].

Apoptosis regulation by p53

Another mechanism of p53 to overcome cellular stress is to induce apoptosis, i.e., one of the modes of “programmed” cell death, involving the elimination of cells that were genetically determined to die. Apoptosis is a physiologic process that occurs ordinarily during development and aging, but can also occur due to immune reactions or upon DNA damage, as a defence mechanism to prevent, for example, cancer [reviewed in [75]]. This process is characterized by a reduction of cellular volume, condensation of chromatin, DNA fragmentation, without inflammation and destruction of plasma membrane until late in the apoptotic process [reviewed in [76]].

The first clue that p53 could have a role in this complex process arose from several studies that correlated the resistance to apoptosis in p53-null mice with tumour progression after irradiation, allowing the understanding that p53 contributes to the induction of apoptosis under stress conditions [77-80]. Interestingly, the role of p53 in apoptosis is evolutionarily conserved in *Drosophila* [81] and *Caenorhabditis elegans* [82], in which p53 curiously does not induce cell cycle arrest, since in these organisms most of the adults cells are postmitotic [reviewed in [67]].

In mammals, apoptosis is a complex process that has two distinct pathways, extrinsic and intrinsic, which culminate with the activation of caspases (cysteine-dependent aspartate-specific proteases) and subsequent apoptosis. The p53 protein participates in these two pathways by transcriptional induction of several target genes, including PUMA (p53 up-regulated modulator of apoptosis), NOXA, Bax (Bcl2-associated X), Bak (Bcl2-antagonist/killer-1), DR5/KILLER, Fas (first apoptosis signal receptor), Bid (BH3 interacting-domain death agonist), among others [reviewed in [83]], as described below.

The extrinsic pathway is activated by the induction of death receptors that belong to the tumour necrosis factor receptor (TNF-R) family, such as Fas/CD95 [84], Dr4 and DR5/KILLER [85]. In response to irradiation, p53 induces the transcription of Fas expression by promoting the trafficking of the receptor from the Golgi complex. Contrary, in response to DNA damage, p53 promotes the transcription of the DR5/KILLER receptor that after binding to its ligand, TRAIL (TNF-related apoptosis-inducing ligand), leads to activation of caspase-8. The receptor activation is followed by its dimerization with recruitment of the intracellular adaptor molecule FADD (Fas-associated protein with death domain) or TRADD (TNFR1-associated death domain), inducing the formation of DISC (death-inducing signalling complex) [reviewed in [86, 87]]. This complex activates the cascade of caspases, starting with caspase-8 and -3 (Figure 4) [reviewed in [67, 83, 88]].

On the other hand, the intrinsic pathway, also known as Bcl-2-regulated apoptotic pathway or mitochondrial pathway, is activated by several stress stimuli [reviewed in [89]]. This designation is due to the crucial role of Bcl-2 (B-cell lymphoma 2) family in this pathway, leading to the release of cytochrome *c* (cyt *c*) from mitochondria [90]. Basically, this family is composed by pro-apoptotic and anti-apoptotic members that have a similar structure in Bcl-2 homology (BH) domains (BH1, BH2, BH3 and BH4). The BH3 domain, present in all members, is essential for the pro-apoptotic functions through the promotion of heterodimerization among Bcl-2 family members. Additionally, the members of Bcl-2 family can be divided into three classes: pro-survival proteins such as Bcl-2, Bcl-xL, Bcl-W and Bcl-B; pro-apoptotic proteins with four BH domains such as Bak, Bax and Bok; or pro-apoptotic proteins with BH3-only domain, such as Bim, PUMA, Bid, Bad, NOXA and Bik. Altogether, these members establish a balance between them, that determines whether or not a cell commits apoptosis [reviewed in [91, 92]].

The activation of PUMA and NOXA by p53 is decisive for the apoptotic process since they mediate p53-dependent apoptosis by binding and antagonizing Bcl-2 anti-apoptotic members, allowing the release of Bax and Bak [93, 94]. Likewise, Bax is inactivated in the cytosol and when activated, direct or indirectly by p53, it is translocated to mitochondria [95]. Conversely, Bak is already in the mitochondrial membrane, even in its inactive form, and when activated, it also allows the release of cyt *c* from mitochondria, as well as Bax (Figure 4) [96].

The initiation of the intrinsic pathway is made by the release of Bax, through inhibition of Bcl-2/Bcl-xL by NOXA or PUMA, or through direct activation of Bax/Bak. [97]. The release of Bax promotes its translocation from cytosol to mitochondria, causing mitochondrial outer membrane permeabilization (MOMP) and release of cyt *c* from mitochondria (Figure 4). After the release, cyt *c* binds to Apaf-1 (Apoptotic protease-activating factor 1) to form the apoptosome complex, activating caspase-9, in the presence of ATP. Subsequently, caspase-9 cleaves caspase-3 and the remaining cascade of caspases (Figure 4). Simultaneously to cyt *c* release, the pro-apoptotic Smac (Second mitochondria-derived activator of caspase) is also released from mitochondria, which deactivate the IAP (proteins-inhibitors of caspases) proteins that block caspase-9 and -3 [reviewed in [67, 89]]. At the end of the caspase cascade, the effector caspases (caspase-3, -6 and -7), activated by initiator caspases (caspase-8 and -9), cleave several intracellular substrates, resulting in cellular modifications that culminate in apoptosis (Figure 4) [reviewed in [88, 98]].

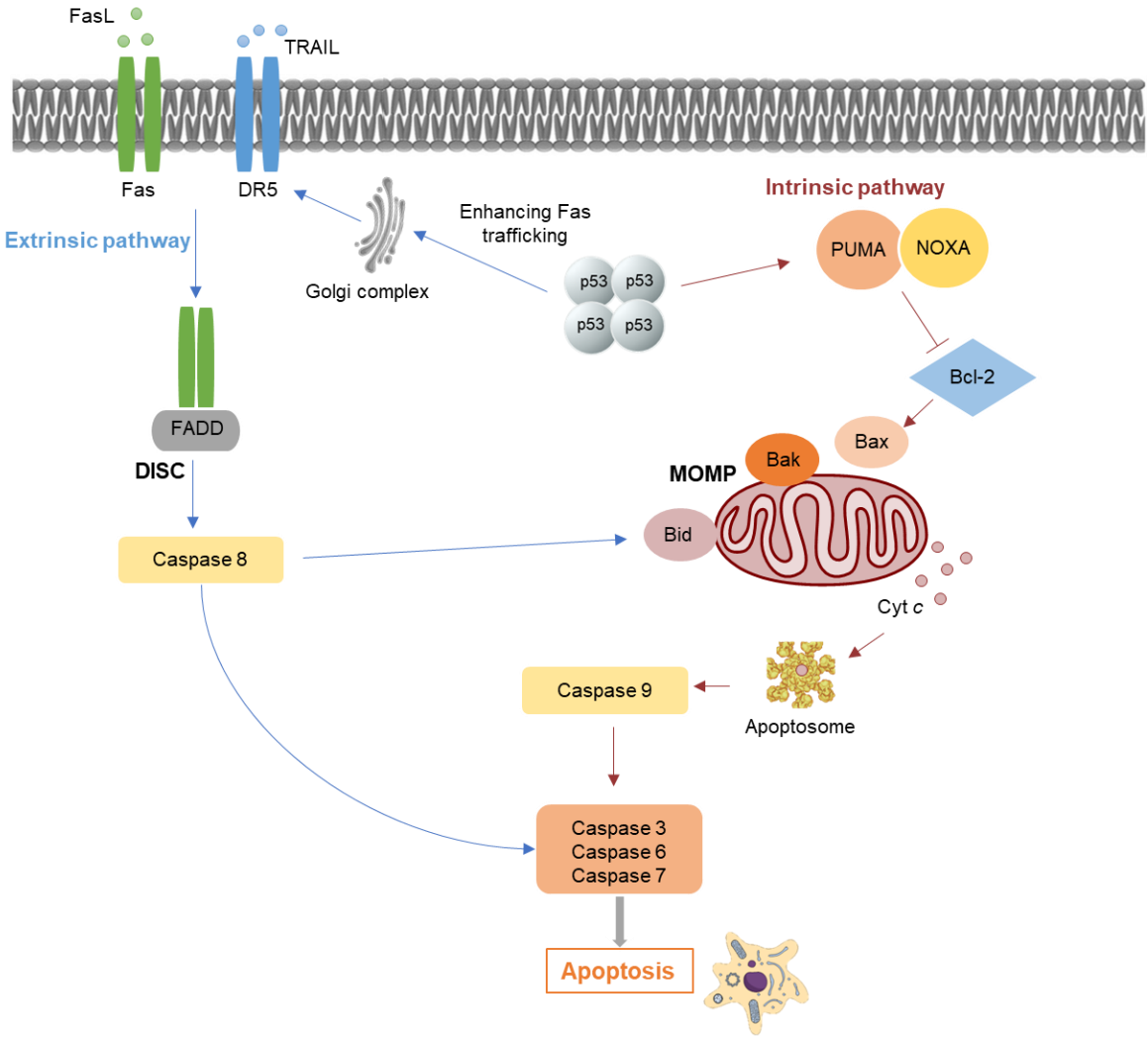

Figure 4 - Regulation of extrinsic and intrinsic pathways of apoptosis by p53. p53 activates death receptors, such Fas and DR5, to initiate the extrinsic pathway (blue arrows). Death ligands bind to its ligands FasL and TRAIL, respectively, inducing its dimerization. Upon Fas or DR5 dimerization, the recruitment of FADD induces the formation of DISC. This complex activates caspase-8 that activates the rest of the caspase cascade (caspase-3, -6, -7) with apoptosis promotion. When activated by p53, caspase-8 can also cleave Bid, promoting its translocation to mitochondria. In the intrinsic pathway (pink arrows), under some stress, p53 is activated, promoting the expression of PUMA and NOXA that blocks Bcl-2 activity. Bcl-2 inhibition leads to the release of Bax, that is translocated to mitochondria and subsequently activated. Bax and Bak cause MOMP and the release of cyt *c*, that binds to Apaf-1, forming the apoptosome complex. This complex activates caspase-9 and the remaining caspase cascade, with apoptosis induction. Fas: first apoptosis signal receptor; FasL: first apoptosis signal ligand; DR5: death receptor 5; TRAIL: TNF-related apoptosis-inducing ligand; FADD: Fas associated protein with death domain; DISC: death-inducing signalling complex; PUMA: p53 upregulated modulator of apoptosis; Bcl-2: B-cell lymphoma 2; Bax: Bcl2-associated X; Bak: Bcl2-antagonist/killer-1; Bid: BH3 interacting-domain death agonist; Cyt *c*: cytochrome *c*; MOMP: mitochondrial outer membrane permeabilization.

Besides the induction of target proteins involved in apoptosis, p53 is also capable of directly activate several proteins, such as Apaf-1 [99] and caspase-6, present at the end of the apoptotic pathway, which had been associated with p53-induced neuronal cell death [100]. Likewise, p53 can activate Bid, that is cleaved by caspase-8, with translocation to the mitochondria, activating Bax and Bak, as well (Figure 4). Bid is the prove that the two apoptotic pathways are not completely independent, being a bridge between them [101]. In addition, p53 can bind to Bcl-2 and Bcl-xL, instead of PUMA or NOXA activation, causing the release of Bax [102].

Moreover, the induction of these targets is mainly due to p53 transcriptional activity, through its DBD, or due to p53 interaction with other proteins [reviewed in [103]]. Survivin is also a p53 target, however in this case p53 supresses survivin activity. Survivin is an IAP that can inhibit apoptosis when overexpressed by interacting with XIAP (X-linked IAP), a cofactor molecule inhibitor of caspase-3 and -7. As expected, survivin appears in a high frequency in p53-mutated tumours, preventing tumour cell death [[104, 105]; reviewed in [106]].

The relevance of p53 in apoptosis is further reinforced by the contribution of the p53-dependent apoptosis to chemotherapy-induced cell death, being associated with chemoresistance in tumours lacking p53 [reviewed in [21]].

Role of p53 in metabolism

Recently, metabolic reprogramming became a hallmark of cancer, based on several studies reporting the need of tumour cells to induce metabolic changes in order to grow, proliferate and survive [reviewed in [8]]. Some of the metabolic changes observed in tumour cells include the fast energy generation, the enhancement of macromolecules biosynthesis and the maintenance of cellular redox status [reviewed in [107]]. The most studied metabolic phenotype of tumour cells is the Warburg effect, also known as aerobic glycolysis, which is characterized by the higher rate of glucose uptake with higher production of lactate. This phenomenon occurs because tumour cells utilize glycolysis for the production of energy, even under normal oxygen levels, in spite of the use of the mitochondrial oxidative phosphorylation (OXPHOS) [108]. Curiously, to date, the Warburg effect is a clinical base for Positron Emission Tomography (PET) imaging, allowing the detection of the primary tumour and regional or distant metastasis, since tumours cells catch more of the glucose analogue ^{18}flurodeoxyglucose (FDG) than normal cells [reviewed in [109]].

The p53 protein is also involved in the regulation of metabolic reprogramming. Indeed, p53 stimulates OXPHOS and inhibits aerobic glycolysis [reviewed in [110]]. The stimulation of OXPHOS by p53 is due to the synthesis of cytochrome *C* oxidative 2 (SCO2), a key enzyme of the Cytochrome *C* complex that catalysis the oxygen-consuming step of mitochondrial respiration (Figure 5) [111]. For the inhibition of aerobic glycolysis, p53 reduces the glucose uptake in the cell by inhibition of the expression of glucose transporters GLUT1 and GLUT4 (Figure 5) [112]. Other ways to reduce aerobic glycolysis is by the degradation of phosphoglycerate mutase (PGM), responsible for catalysing the rearrangement of the phosphate group position on the 3-phosphoglycerate molecule (Figure 5) [113]. Also, the upregulation of hexokinase 2 (HK2) and *TP53*-induced glycolysis and apoptosis regulator (TIGAR) decrease the glycolytic flux through the reduction of glucose-6-phosphate and fructose-2,6-biphosphate expression, respectively, important in the activation of rate-limiting steps of glycolysis (Figure 5) [114]. The p53 downregulation of monocarboxylate transporter 1 (MCT1) suppresses lactate secretion, at the end of the glycolytic pathway, also stimulating the shift to OXPHOS for energy generation, as a consequence of the accumulation of pyruvate that cannot be converted to lactate (Figure 5) [reviewed in [110]].

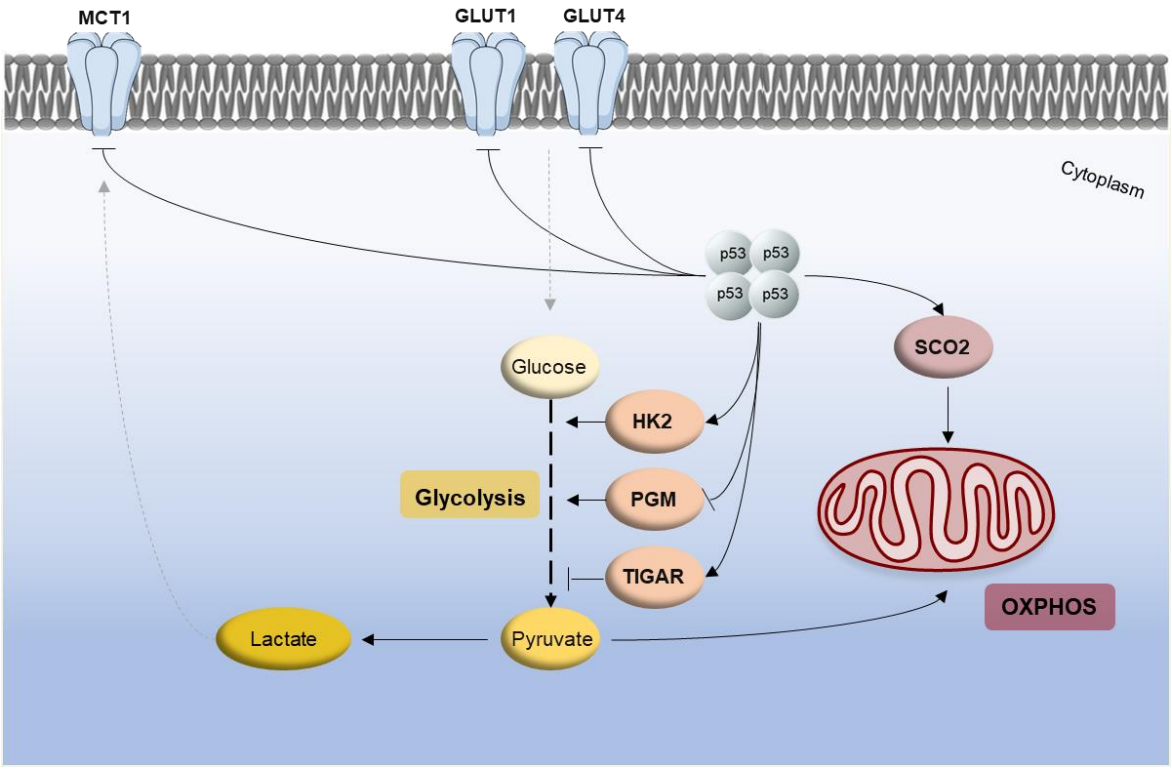

Figure 5 – Regulation of metabolic reprogramming by p53. p53 inhibits the glucose uptake through the inhibition of GLUT1 and GLUT4, the first step of aerobic glycolysis. Throughout the glycolysis pathway, p53 induces the expression of HK2 and TIGAR, and inhibit the expression of PGM that decreases the glycolytic flux. At the end of the glycolytic pathway, the lactate secretion is suppressed by p53 through MCT1 downregulation. As a consequence of the suppression of secretion of lactate, pyruvate accumulation promotes the shift to OXPHOS. At the same time, p53 also induces the expression of SCO2 to stimulate OXPHOS. GLUT: glucose transporter; HK2: Hexokinase 2; PGM: phosphoglycerate mutase; TIGAR: *TP53*-induced glycolysis and apoptosis regulator; MCT1: monocarboxylate transporter 1; OXPHOS: oxidative phosphorylation; SCO2: synthesis of cytochrome *C* oxidative 2.

Therefore, the inhibition of glycolysis progression through modulation of several proteins obligates tumour cells to use OXPHOS for energy generation [reviewed in [115, 116]]. Altogether, several evidences demonstrated that p53 is also a promising target in a metabolic context, mainly associated with glycolysis and OXPHOS inhibitors to achieve a synergistic effect [reviewed in [110]].

1.2.3 Impaired p53 in cancer

In this chapter, it has been highlighted the relevance of p53 in many cellular processes, showing to be a multifunctional protein. In the absence of a p53 normal

activity, some stress conditions in cells are unable of being solved, since many cellular processes are dependent on p53 target genes [reviewed in [50]].

p53 inhibition by MDM2 and MDMX in cancer

Under unstressed conditions, p53 is maintained at low expression levels by diverse negative regulators, such as MDM2, MDMX, PIRH2 and COP1 [reviewed in [52, 89, 117]].

Among its negative regulators, MDM2, also known as HMD2 for its human analogue, is the major negative regulator, maintaining the p53 stability and activity. MDM2, an oncoprotein and E3 ubiquitin ligase with 491 amino acids, was identified in a double minute chromosome from a murine tumourigenic cell line, which controls the p53 expression levels by promoting p53 proteasomal degradation [118]. This control can be done through two different mechanisms: the *N*-terminal TAD of MDM2, namely its hydrophobic pocket, can directly bind to phenylalanine 19, tryptophan 23, leucine 26 residues of p53 *N*-terminal TAD, promoting the p53 translocation from the nucleus to the cytoplasm, with suppression of p53 transcriptional activity. On the other hand, MDM2 presents a *C*-terminal RING-finger domain, containing E3 ligase activity, that promotes proteasomal-mediated degradation by the ubiquitination of specific lysine residues on the *C*-terminal end of p53. Therefore, in the absence of cellular stress, increased MDM2 levels result in low p53 levels (Figure 6) [reviewed in [52, 117, 119]]. Moreover, previous studies showed that MDM2-p53 interaction occurs through a feedback loop, in which MDM2 regulates p53 levels and activity, while p53 regulates MDM2 expression [120].

The MDM2 homologue, MDMX, also known as MDM4 and HDMX/4 for its human analogue, can also bind to the *N*-terminal TAD of p53, blocking its transcriptional activity (Figure 6) [121]. MDMX lacks E3 ligase activity, being unable to directly promote the p53 degradation. Despite this, MDMX can bind to MDM2, through interaction of its RING-RING domains, enhancing the nuclear degradation of p53 and the MDMX ubiquitination by MDM2 (Figure 6) [reviewed in [49, 119]].

The ubiquitination of p53 by MDM2 can occur by two distinct ways, depending on MDM2 expression levels. In case of low levels, MDM2 as a homotetramer induces monoubiquitination of p53, which promotes the export of p53 to the cytoplasm for proteasomal ubiquitination (Figure 6). In case of high levels, MDM2 form a heterotetramer with MDMX that mediates p53 poly-ubiquitination, and the subsequent p53 nuclear

degradation (Figure 6). In both cases, MDM2/MDMX interaction endorses the expression of basal levels of p53 (Figure 6) [122].

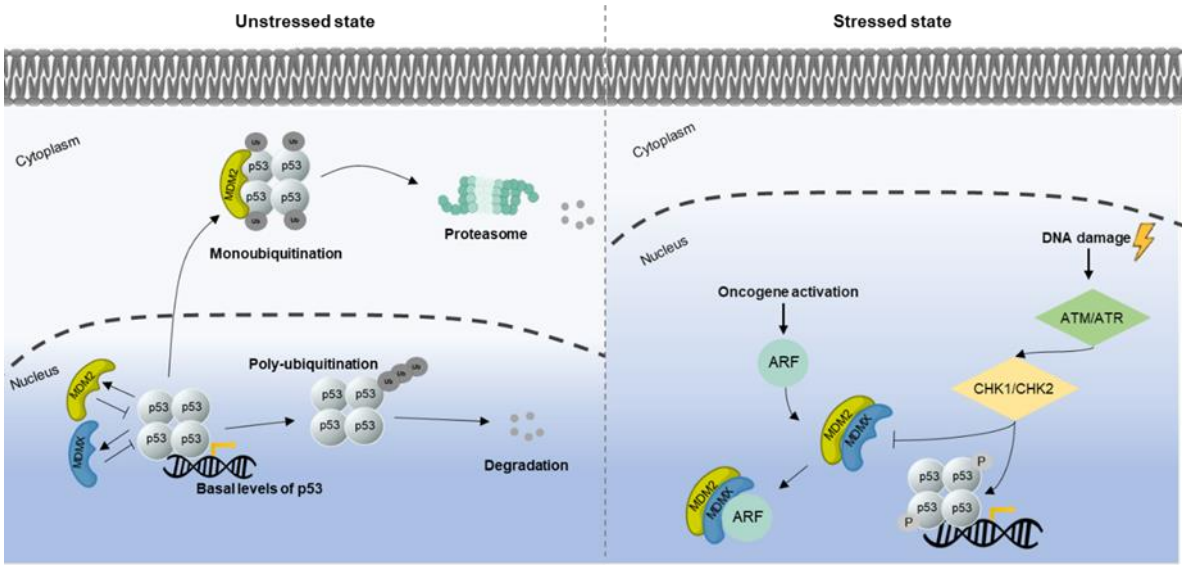

Figure 6 - Regulation of p53 by MDM2 and MDMX, in unstressed and stressed cellular conditions. Under unstressed conditions: MDM2 and MDMX will promote p53 degradation, either by monoubiquitination or poly-ubiquitination, preventing high expression levels of p53. The choice between mono or poly-ubiquitination depends on the low or high levels of MDM2 expression, respectively. In case of monoubiquitination, MDM2 promotes the export of p53 to the cytoplasm, for proteasomal ubiquitination. In case of poly-ubiquitination, p53 is degraded in the nucleus by MDM2 and MDMX. **Under stress conditions:** p53 needs to be activated to promote the transcription of target genes to maintain cellular homeostasis. Oncogene activation leads to the activation of ARF that repress MDMs, allowing p53 activation and transcriptional activity. DNA damage activates ATR/ATM kinases, which in turn activates Chk1/Chk2 kinases, responsible for the inhibition of p53 repression by MDMs. MDM2/X: murine double minute 2/X; Ub: Ubiquitination; ARF: Alternative Reading Frame; ATM: Ataxia Telangiectasia Mutated; ATR: Ataxia Telangiectasia and Rad3 related; Chk1/2: Cell Cycle Checkpoint Kinase 1/2; P: phosphorylation.

The stabilization and activation of p53 occurs in response to several extrinsic and intrinsic stress signals, such as oncogene activation, genotoxic stress, ribosomal stress and deficiency of oxygen or other nutrients, through the disruption of the p53-MDM2/MDMX interactions [reviewed in [52, 117]]. In case of oncogene activation, ARF (alternative reading frame) tumour suppressor protein ($p14^{ARF}$), a negative regulator of MDM2 and MDMX activity, allows the MDM2 proteasomal degradation with consequent stabilization and activation of p53 (Figure 6). Besides proteasomal degradation of MDM2, $p14^{ARF}$ also prevents the nuclear export of MDM2 [123]. However, in case of DNA damage, ATM and ATR kinases are activated as a response to DNA damage, which

subsequently activates Chk2 and Chk1, respectively (Figure 6). These four kinases are responsible for the p53 phosphorylation, decreasing the affinity for MDM2 and concomitantly increasing the affinity for p300/CBP, since these proteins have overlapping binding sites within the *N*-terminal region of p53 (Figure 6) [124-126]. These two mechanisms guarantee p53 activation and subsequent expression of p53 target genes that promote the restoration of cellular homeostasis in normal cells.

In tumour cells with wtp53, p53 inactivation is made by abrogation of its signalling pathway. Interesting, epidemiological studies reported a strong association of HPV with the development of cervical tumours [127]. In fact, Human Papilloma Virus (HPV) produces an E6 viral oncoprotein able to bind to wtp53, inducing its degradation [128]. Despite this, MDM2 and MDMX proteins are the most prevalent forms of inactivation of wtp53. This inactivation can occur mainly through amplification of *MDM2/X* genes, elevated *MDM2/X* expression levels or through activation of other pathways. Consequently, an increased activity of these p53 endogenous modulators results in a higher p53 degradation, which leads to the loss of its normal activity. In fact, *in vivo* studies showed that overexpression of MDMs in transgenic mice leads to the development of spontaneous tumours, revealing its oncogenic functions [[129, 130]; reviewed in [131]].

The frequency of MDM2 and MDMX overexpression in different types of tumours is not related, demonstrating non-redundant functions between these two proteins [reviewed in [132]]. The most frequent types of adult solid tumours that present increased activity of MDM2 are osteosarcoma, glioblastoma and prostate cancer. On the other hand, MDMX overexpression is mainly associated with melanoma, retinoblastoma, hepatocellular carcinoma and breast cancer [133-136]. Regarding haematological malignancies, MDM2/X overexpression appears in the majority of the cases as the cause of p53 impairment [reviewed in [137]]. In addition, MDM2/X overexpression is also highly frequent in paediatric tumours, such as sarcomas, gliomas and leukaemias [reviewed in [59]].

Functional activities of Mutant p53 and Li-Fraumeni Syndrome

TP53 is one of the most mutated gene in human tumours both in germline and somatic contexts [138, 139]. Although there are different types of alterations throughout the *TP53* gene, the missense mutations, defined by the substitution of a single amino acid in a codon, are the most frequent type of alteration found in human tumours. In fact, missense mutations lead to the loss of normal p53 transcriptional activity, particularly of its

tumour suppressor function [reviewed in [140]]. Furthermore, the prolonged half-life of mutp53 and its higher stability, when compared to wtp53, is caused by its incapacity to act as a transcriptional factor, affecting the regulatory feedback loop between MDM2 and p53 mentioned above [141]. Moreover, more than 90% of the missense mutations occur in the DBD of p53, particularly in six hotspot residues (175, 245, 248, 249, 273, 282), resulting in the expression of full-length mutp53 proteins (Figure 7) [reviewed in [140, 142]]. Additionally, missense mutations can be classified as DNA contact (e.g. R248Q, R248W, R273C, R273H, R280K and R282W) or as structural (e.g. R175H, Y220C, G245S, G245D and R249S), if it only affects the p53 DNA binding ability or if it disrupts its conformation, respectively [reviewed in [143, 144]].

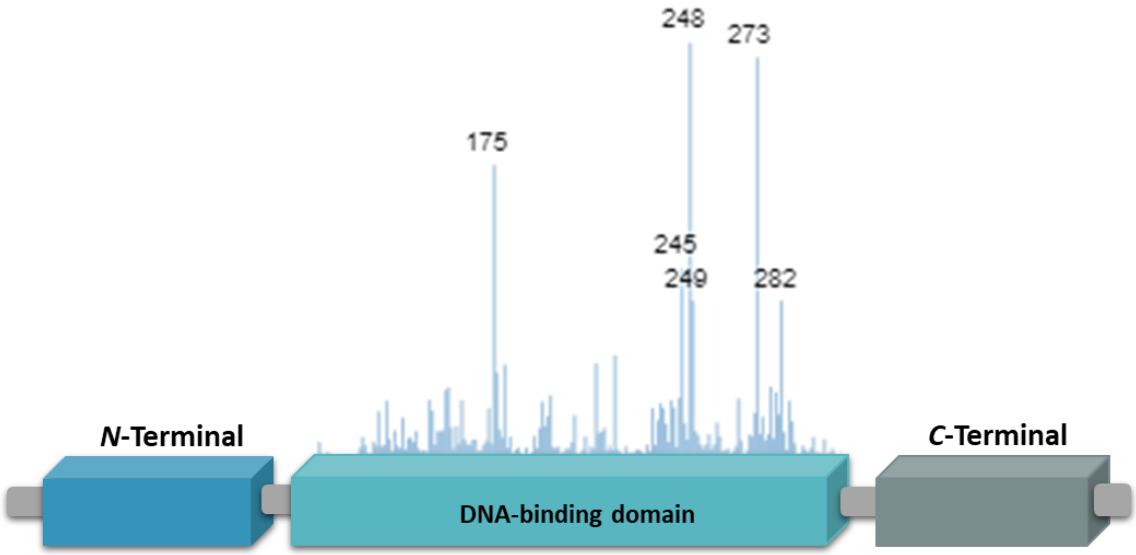

Figure 7 - Graphical representation of the most prevalent somatic missense mutations in the *TP53* gene. Each number indicate each "hotspot" residue and each line indicate the relative frequency of tumour-associated mutations for each residue (high frequency - 7%) according to the *TP53* mutation database [145].

Germline mutations in *TP53* gene cause Li-Fraumeni Syndrome (LFS), a cancer hereditary syndrome. Particularly, germline mutations in p53 have been identified in approximately 70% of LFS patients [146]. LFS was first described in 1969 by Li and Fraumeni and is classified as a familial autosomal dominant cancer syndrome described by early onset of multiple tumours [147, 148]. However, only in 1990, the *TP53* gene was linked to the syndrome, in which the most three common mutated residues are 248, 273 and 337 [149]. The incidence of this syndrome is 1 case per 5000 individuals, and the most types of LFS-associated tumours include osteosarcomas, soft-tissue sarcomas, breast and brain tumours, adrenocortical carcinomas, and leukaemia. LFS individuals

have increased probability to develop cancer before age 30 and they also have an increased risk to develop second tumours, in which some of them are associated with ionizing radiation-treatment of the first malignancy [150]. In these cases, as in other syndromes, individuals from families with a known *TP53* mutation should be submitted to *TP53* genetic tests or to follow the LFS criteria – The Chrompet criteria – for an early diagnosis [reviewed in [151]].

Contrary to germline mutations, somatic *TP53* mutations in sporadic tumours mainly occur by exposure to carcinogens such as ultraviolet radiation and tobacco smoke [reviewed in [152]]. The tumour site distribution related to somatic mutations include higher percentage of colon, breast, ovarian and head and neck carcinomas. Despite these differences, in the majority of germline and sporadic tumours, after mutation in one *TP53* allele, the second wt allele is lost at a later stage of the disease, normally through the loss of heterozygosity (LOH), resulting in the expression of mutant p53 only [reviewed in [142]]. In fact, p53 missense mutations have the ability to induce oncogenic functions, commonly related to more aggressive tumours with poor prognosis, in which unfavourable therapeutic responses are commonly observed [153, 154].

One mechanism of mutp53 to induce oncogenic activities is by a dominant negative effect (DNE) on the remaining wt allele [reviewed in [155]]. Upon missense mutation in one allele of p53, it is generated a DN mutant that can inactivate the co-expressed *wt* allele, resulting in weaker tumour suppression. Indeed, this might be the reason for the increased cancer susceptibility of LFS patients [156]. Furthermore, several *in vivo* studies showed that mutp53 is capable to inhibit the activity of wtp53, either through the formation of heterotetramers with mutp53 or through the inhibition of w*t*p53 binding to its target gene promoters, preventing wtp53 transcriptional activity [157]. However, these DNE depend on several factors, such as the required ratio (3:1) of mutp53 to wtp53 within the heterotetramers [158], the conformation of p53 mutation, the DNA binding site and the DNA affinity [157, 159]. In fact, the DNE is only observed when tumour cells have high levels of mutp53 expression levels, indicating that these effects are critical to the treatment approach, since radiation therapy and chemotherapy resistance are associated with higher levels of mutp53. Thus, therapeutic approaches that lead to a reduction of mutp53 levels would improve the therapeutic response [reviewed in [50, 142]].

Additionally to DN activities of mutp53, in 1993 emerged the gain-of-function (GOF) concept, indicating an enhancement of tumour progression. These conclusions result from *in vitro* studies demonstrating that transfection of different p53 mutants to p53-

null tumour cells resulted in the decrease of expression of p53 target genes [160]. However, when mutp53 were silenced by small interfering RNA, the expression of p53 targets increased, with the consequent suppression of human bladder tumour cells growth [161]. Supporting these observations, *in vivo* studies showed that knocking mice expressing the p53 GOF mutants develop more aggressive tumours with metastatic profile than p53-null- and wtp53-expressing mice [162, 163].

Furthermore, over the last years, several studies have unveiled how some mutations are able to confer oncogenic abilities. Firstly, p53 mutations can regulate the expression of unregulated/repressed genes by wtp53, including multidrug resistance 1 (*MDR1*) gene, conferring drug resistance to mutp53-expressing tumour cells [164], and also some proteins involved in apoptosis and angiogenesis, such as PCNA, Fas and vascular endothelial growth factor (VEGF) [165-167]. Secondly, mutp53 may inhibit the transcriptional activity of other p53 family members, including p63 and p73, blocking their capacity to suppress growth and to induce apoptosis [168, 169]. Lastly, these mutations can also inhibit DNA repair genes, for example, the interaction of mutp53 R248W with the Mrc11-Rad5-NBS1 (MRN) complex, blocking the recruitment of ATM kinase, with subsequent genomic instability [170]. Altogether, these GOF functions promote tumour progression through stimulation of several hallmarks of cancer, such as enhancement of angiogenesis, genomic instability, invasion, migration, cell proliferation, cell death resistance and chemoresistance (Figure 1). For all these reasons, the genetic signatures and clinical characteristics are of extreme relevance to personalize the best therapeutic approach for each patient. In fact, according to the p53 status, it is possible to stratify patients based on different types of therapies that target p53, which will allow improved patient outcomes [reviewed in [50, 140]].

1.2.4 Therapeutic strategies for targeting p53 in cancer treatment

Nowadays, precision medicine is a “hot topic” in cancer treatment. Additionally, the restoration of p53 normal activity represents a promising targeted anticancer therapeutic strategy. In fact, as referred above, cumulated data have shown a regression of established tumours upon restoration of p53 activity, proving the enormous benefit of the normal p53 function to block the development and progression of tumours [171-173]. As such, over the last years, several p53-target therapies have emerged, including gene therapy, vaccines to eliminate mutp53-expressing tumour cells, inhibition of the MDM-p53 interaction, and reactivation of wt-like function to mutp53 [reviewed in [174]].

Gene therapy

Adenoviral delivery of wtp53 (Ad-p53) into tumour cells is known as gene therapy and allows to rescue the p53 activity in cancer [175]. This strategy has been tested in China with Gendicine (Shenzhen Sibiono Genetech, China), reaching to phase IV clinical trials for the treatment of several recurrent solid tumours [176-178]. Previous *in vitro* and *in vivo* studies have shown marked apoptotic effects in tumour cells, a good regression response in diverse types of tumours without toxic effects on normal cells [179-182]. In a clinical study, the combination of Gendicine and radiation therapy increased the complete response, 5-year overall survival and 5-year disease-free survival rates [178]. Besides these remarkable responses, this therapy is only used in China, since Food and Drug Administration (FDA) and European Medicines Agency (EMA) did not approve this therapeutic approach. The major restriction of this strategy is the incapacity of the adenovirus to reach every tumour cells in patients, due to limitations in virus delivery and the reduction of the infectivity of the adenovirus by host antibodies [reviewed in [183]].

p53-based vaccines

The differences between the low expression levels of wtp53-expressing normal cells compared to the high levels of mutp53-expressing tumour cells provides the basis for p53 immunotherapy, since p53 is also considered a promising target antigen [reviewed in [174, 184]]. Two distinct vaccines entered into phase I/II clinical trials, p53-SLP (a p53 synthetic long peptide vaccine) [185] and INGN-225 (a p53-modified adenovirus-transduced dendritic cell vaccine) [186], both presenting specific anti-p53 immune responses in 90% of metastatic colorectal cancer patients and 42% of small cell lung cancer patients, respectively. These two vaccines were considered well tolerated, giving the opportunity for the initiation of new clinical trials to improve response rates and combine with other therapeutic options.

Elimination of mutant p53

The elimination of mutp53 represents an effective strategy to block its oncogenic effects, which, prevents DNE or GOF in tumour cells, with subsequent inhibition of tumour cell growth. Some small molecules identified, such as 17AAG and SAHA, can degrade mutp53 by inhibition of HSP90 (chaperone that interacts with p53) or of histone deacetylase 6 (HDCA6) that upregulate HSP90, respectively [141, 187]. Other drugs such as arsenic trioxide and gambogic acid can also degrade mutp53 by ubiquitination and subsequently proteasomal degradation [188, 189].

Activation of wtp53 functions

One of the most promising therapeutic approaches to target p53 consists in small molecules capable to release p53 from MDM2 in wtp53-expressing tumour cells. There are several approaches to inhibit the interaction between these proteins, including blocking the interaction between them, downregulating MDM2 expression, and modulating the E3 ubiquitin ligase activity of MDM2 [reviewed in [137]]. The first group of small molecules developed was Nutlin cis-imidazolines analogues, discovered by Vassilev and colleagues in 2004. Nutlin molecules (Nutlin-1, Nutlin-2 and Nutlin-3) bind to the hydrophobic pocket of MDM2, that normally binds to the three critical amino acid residues of p53 TAD (*Phe19, Trp23* and *Leu26*), blocking the interaction between the two proteins with subsequent induction of p53 accumulation and transcriptional activity (Table 3) [190]. The optimization of this class of compounds resulted in the development of another potent MDM2-inhibitor, RG7112. RG7112 was the first MDM2-antagonist undergoing clinical trials, completing phase I study in diverse types of tumours and in combination therapies (doxorubicin and cytarabine) [[191-194]; reviewed in [137]]. Despite its high efficacy as MDM2-inhibitor, RG7112 was also associated with haematological toxicity in a phase I study on patients with advanced solid tumours [191]. Another small molecule of the same group is RG7388, also known as idasanutlin [195]. So far, RG7388 is the MDM2-inhibitor that has progressed further in clinical trials, already recruiting patients with relapsed/refractory acute myeloid leukaemia for a phase III study (NCT02545283).

During the last years, other classes of highly efficient inhibitors of the MDM2-p53 interaction were discovered, including spiro-oxindole and piperidonone [reviewed in [52, 183]]. Recently, our group identified three compounds capable to inhibit the MDM2-p53 interaction, pyranoxanthone 1 [196], oxazolopyrrolidone 3a [197] and prenylchalcone 2 [198], demonstrating antitumour activity in colon tumour cells (Table 3).

Curiously, a small molecule discovered by Selivanova and colleagues, RITA (reactivation of p53 and induction of tumour cell apoptosis) was firstly described as inhibitor of the p53-MDM2 interaction [199]. Nevertheless, recently data have abolished this assumption, demonstrating that the activity of RITA was not dependent on wt or mutp53, but rather on DNA damage [200].

In addition, some small molecules were also designed to target MDMX, being particularly relevant against some types of tumours with overexpression of MDMX [reviewed in [183, 201]]. The first small molecule designed to target MDMX was SJ-172550, an imidazoline derivative that proved to inhibit MDMX, and synergistically with nultin-3 triggers apoptosis in some types of tumours [202, 203]. Other small molecules

were developed as MDMX-inhibitor, such as benzofuroxan derivatives and pseudourea derivatives (Table 3) [reviewed in [52]].

In 2012, further studies led Vassilev and colleagues to discover the dual MDM2-MDMX inhibitors RO-2443 and RO-5963, which can overcome the apoptotic resistance to nutlin-3 in cells that overexpress the MDMX protein [206]. Moreover, our group also identified two other promising small molecule dual inhibitors of the MDM2/MDMX-p53 interactions , OXAZ-1 (a tryptophanol-derived oxazolopiperidone; [207]) and DIMP53-1 (a tryptophanol-derived oxazoloisoindolinone; [208]) (Table 3).

Table 3 – Small molecules that activate wtp53 function.

Molecule	Class	Mechanism of action	Reference
Nutlin	Imidazoline	MDM2-p53 interaction	[190]
RG7112*			[192, 193]
RG7388 (idasanutlin)*	Pyrrolidine		[195]
MI-773*	Spiro-oxindole		[209]
AMG232*	Piperidone		[210, 211]
Pyranoxanthone 1	Pyranoxanthone		[196]
Oxazolopyrrolidone 3a	Oxazolopyrrolidone		[197]
Prenylchalcone 2	Prenylchalcone		[198]
SJ-172550	Imidazoline	MDMX-p53 interaction	[202, 203]
XI-006	Benzofuroxan		[204]
XI-011	Pseudourea		[205]
RO-2443	Indolyl hydantoin	Dual MDM2-MDMX inhibitors	[206]
RO-5963			
OXAZ-1	Tryptophanol-derived oxazolopiperidone lactam		[207]
DIMP53-1	Tryptophanol-derived oxazoloisoindolinone		[208]

* Coumpounds under clinical trials.

In mutp53-expressing tumour cells, these compounds have been explored in a context of cyclotherapy, in order to protect normal cells from cytotoxic drugs. In fact, low doses of MDM-p53 inhibitors can induce cell cycle arrest in a p53-dependent manner, more precisely an arrest in G1-phase, avoiding, at the same time, the toxic effects caused by these compounds when administrated at high doses. Since mutp53-expressing tumour cells have a defective G1-arrest checkpoint, continuing to divide, guarantying that in the presence of cytotoxic agents, targeting S- or M-phases, apoptosis is triggered only in tumour cells [reviewed in [183, 212]]. In some *in vitro* studies, low doses of nutlin-3 with taxol or gemcitabine resulted in apoptosis of tumour cells and in a protective effect of normal tissues [213]. Although cyclotherapy seems to be a promising approach, controversial results have been obtained in *in vivo* studies. Despite this, it was shown that the pre-treatment with nutlin-3 protected mice from neutropenia induced by the Polo-Like-Kinase-1 (PLK-1) inhibitor BI2536 [214].

Restoration of wt-like p53 function to mutp53

Certain types of cancer, such as high-grade serous ovarian carcinomas and pancreatic adenocarcinoma, have a high *TP53* mutation rate (over 70% to 97%). For these cancers, the use of compounds that restore wtp53-like function to mutp53 (called reactivators) shows to be an encouraging anticancer approach [215, 216].

The first mutp53 reactivators identified through high-throughput screening of chemical libraries was the alkaloid ellipticine [217], its derivative 9-hydroxy ellipticine [218] and WR1065 (restoration of mutp53 V272) [219]. Despite their antiproliferative effects, these compounds also exhibit some toxic effects in normal cells [217-219]. Other small molecules were thereafter identified, including styrlquinazoline, CP31398 [220] and its derivative STIMA-1 [221], maleimide-derived molecule MIRA-1 (mutant p53-dependent induction of rapid apoptosis) and its derivative MIRA-3 [222], and SCH52074 [223]. Although these compounds induce tumour cell death, all of them present high toxicity in normal cells (Table 4).

Another small molecule is PhiKan083, a pyrazole that selectively binds to a unique pocket of mutp53 Y220C, increasing its melting temperature and decreasing its denaturation rate [224]. Later, its derivative PK7088 was identified, demonstrating to be also a reactivator of mutp53 Y220C but, conversely to PhiKan083 with *in vitro* antitumour activity [224]. Two additional reactivators of mutp53 Y220C, 2-sulfonylpyrimidine PK1100 and its derivatives PK11007 and PK11010 [225], were also discovered, but more recent studies showed their independent mutp53 activity (Table 4) [225].

Other two small molecules were specifically identified as reactivators of mutp53 R175H, the thiosemicarbazone NSC319726 [226] and chetomin (CTM) [227], with *in vitro* and *in vivo* antitumour activity (Table 4). However, the mechanism of action of NSC319726 is still controversial [226, 227].

Furthermore, COTI-2, also a thiosemicarbazone, demonstrated to have *in vitro* and *in vivo* antitumour activity against multiple cancer types, being in phase I clinical trial for gynecologic malignancies and head and neck squamous cell carcinoma patients (NCT02433626) [228]. In addition, with a similar activity to COTI-2, the compound p53R3, a quinazoline, was identified as reactivator of mutp53 R175H and R273H (Table 4) [229].

The most promising small molecules in this category are PRIMA-1 [230] and its derivative PRIMA-1^{MET} (APR-246) [221]. Although the first reports of PRIMA-1 had shown its ability to only reactivate mutp53 R175H and mutp53 R273H [230], recent works have demonstrated that PRIMA-1 is also able to reactivate other mutp53 forms. *In vivo* studies with PRIMA-1 demonstrated its antitumour activity without toxicity in normal cells, either alone or in combination with conventional chemotherapeutics, such as cisplatin [230]. Moreover, *in vivo* antitumour activity was also observed with its optimized derivative PRIMA-1^{MET} [231], that already completed phase I clinical trial in patients with AML and prostate carcinoma (NCT00900614). Besides the remarkable responses with a reduction of 20% of blast in one AML patient through the induction of cell-cycle arrest and apoptosis, PRIMA-1^{MET} was also well tolerated [232]. After these results, new clinical trials are ongoing, either alone or in combination in different types of tumours (NCT00900614, NCT03072043, NCT03268382, NCT03391050 and NCT02999893) (Table 4).

Recently our group, using a yeast-based screening assay, also identified a reactivator of mutp53, the small molecule SLMP53-1, a tryptophanol-derived oxazoloisoindolinone. SLMP53-1 reestablished the mutp53 R280K DNA-binding ability and, subsequently, its wt-like transcriptional activity. Additionally, it showed to be a non-genotoxic compound, with encouraging *in vivo* antitumour activity, without apparent toxic side effects, (Table 4) [233].

Table 4 – Small molecules reactivators of mutp53.

Molecule	Mutant	Class	Ref.
Ellipticine	R175H / R248W / R273H	Alkaloid	[217]
9-hydroxy ellipticine	R175H / R273H		[218]
WR1065	V272	Aminothiol	[219]
CP31398	R249S / R273H	Styrlquinazoline	[220]
STIMA-1	R175H / R273H		[221]
MIRA-1 / MIRA-3	R175H / R248W / R248Q	Maleimide	[222]
SCH52074	R273H / R249S	Propanediamine	[223]
PhiKan083	Y220C	Pyrazole	[224]
PK7088	Y220C		[225]
PK1100	Y220C	2-sulfonylpyrimidin	
PK1107	Y220C		
PK11010	Y220C		
NSC319726	R175H	Thiosemicarbazone	[226]
COTI-2*	R175H		[228]
Chetomin	R175H	Epidithiodioxopiperazine	[227]
p53R3	R175H / R273H	Quinazoline	[229]
PRIMA-1	R175H / R273H / R280K	Quinucidinone	[230]
PRIMA-1^{MET} (APR-246)*	R175H / R273H		[231]
SLMP53-1	R280K	Tryptophanol-derived oxazoloisoindolinone	[233]

* Compounds under clinical trials.

Despite the variety of small molecules available, only few entered into clinical trials. In fact, targeting p53 has shown to be a therapeutic challenge, mostly due to: I) the complexity of the p53 pathway [reviewed in [174]], II) the lack of tumour cell death

response to p53-targeted therapies in low-grade tumours with low-level of oncogenic signals [234, 235], III) the development of resistance to p53-targeted therapies by inactivation of $p14^{ARF}$ [171] or through the acquisition of p53 mutations in wtp53-expressing tumour cells [236], and IV) metabolic adaptations [111]. As such, new strategies to achieve an improved p53-based therapeutic response continue to be required, including a better selection of p53-based therapies, personalized to each patient based on the p53 status in tumours. Additionally, the identification of new compounds with improved efficacy is also extremely important to circumvent the drawbacks described for the currently available p53 reactivators [reviewed in [52, 174]].

1.3 Yeast models to search for potential p53 reactivators

Several cell models have been used to study p53. Among them, the yeast *Saccharomyces cerevisiae* has been a valuable tool to understand the biology and pharmacology of p53. In fact, there are various advantages in the use of this model, including its short generation time, easiness and low cost of manipulation, and compliance to genetic manipulation [reviewed in [237, 238]].

Despite its higher simplicity as cell system, *S. cerevisiae* is an eukaryotic organism with a high degree of conservation of the transcriptional machinery with human cells, allowing the study of major functions of p53, particularly in cell cycle and apoptosis [reviewed in [239]]. Additionally, for the particular case of p53, no orthologue genes have been identified in this organism, which has enabled the study of human p53 protein activity by heterologous expression in *S. cerevisiae,* without the interference of similar proteins that would hinder the analysis [reviewed in [238]].

The studies of p53 in yeast started with the simple proof that its expression, as well as its endogenous regulators, confer similar phenotypes between yeast and human cells [196, 240]. In the last years, many yeast-cell based assays were developed for functional and pharmacological studies (Figure 8).

The first yeast-cell based assay to study p53 was developed by Ishioka and colleagues, in 1993, for the functional analysis of separated alleles in yeast (FASAY) assay. This assay intends to identify the p53 status in tumour cells, through the extraction of p53 from tumour tissue samples. In this manner, the first FASAY assay used a *HIS3*-based reporter system, in which yeast clones with wtp53 (His+) grew in plates without histidine, while yeast clones with mutp53 (His-) did not grow [241]. The colorimetric FASAY was developed in 1995, in which the *HIS3*-based reporter system was replaced by

an *ADE2*-based system, evaluating wt and mutp53 yeast clones by its white and red colour in plates, respectively (Figure 8) [242]. This FASAY version allowed confirming that some *TP53* mutations promote partial loss of the p53 transactivation function [242]. Afterward, other FASAY assays were developed to circumvent some percentage of false positives verified in colorimetric FASAY [reviewed in [243]].

One of the most used yeast assays is the yeast two-hybrid (Y2H) that allows the identification of protein-protein interactions. The Y2H assay also involves a reporter system, but in this case each protein of interest is fused to one half of a transcriptional factor, that if reconstituted leads to the transcription of a reporter gene (Figure 8) [244]. This assay was also used to study MDM2 and MDMX interactions with each other or with p53 [245]. Furthermore, Vidal and colleagues [246] developed a reverse Y2H assay to screen for inhibitors of protein-protein interactions, namely of the MDM-p53 interaction. In this case, cell growth only occurs if the protein-protein interaction is disrupted due to interactor inhibitors.

More recently, the dual-luciferase functional yeast assay was developed [247]. This transactivation assay uses a luciferase reporter gene that contains upstream to the minimal promoter (pCYC1) a p53RE, which promotes the transcription and synthesis of a luciferase gene, if wtp53 is activated [247]. In this manner, light emitted by luciferase is proportional to p53 transcriptional activity, allowing the screening of p53 small molecule modulators. Despite these advantages, this is an artificial system more expensive than other assays (Figure 8) [247].

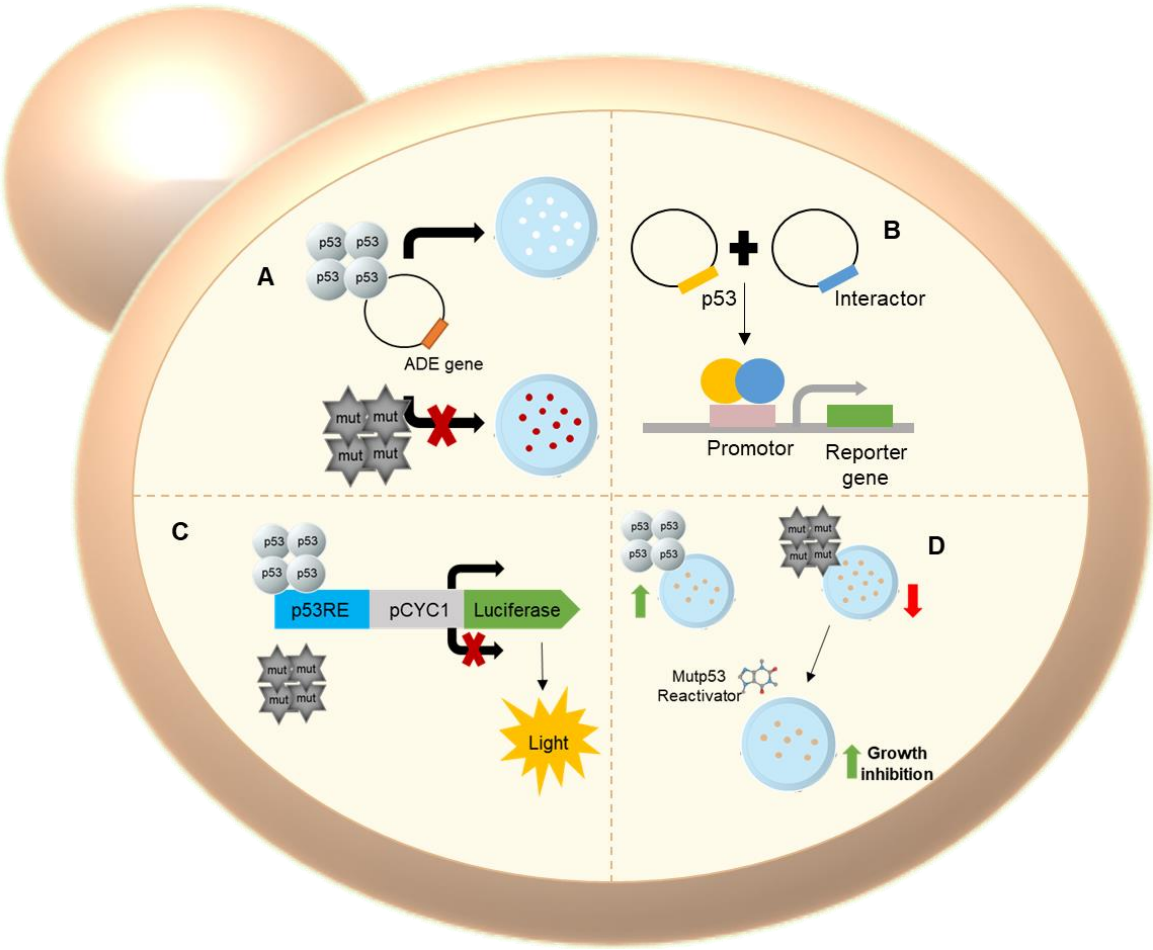

Figure 8 - Schematic representation of the four yeast assays used for pharmacological and functional studies of p53. A) The colorimetric FASAY assay allows evaluating the existence of yeast clones with wtp53 or mutp53 by the colony colour in the plates. Wtp53 clones present a white colour due to the transcription of the reporter gene. On the other hand, yeast with mutp53 cannot transcript the reporter gene, presenting red clones. **B)** The Y2H assay allows the elucidation of protein-protein interactions. In this case, it is possible to test the interaction of p53 with its endogenous regulators or p63 and p73. For this, two plasmids are needed to express the two proteins of interest. If these two proteins interact, they reconstitute a promoter that subsequently promotes the expression of a reporter gene. **C)** In the dual-luciferase functional assay, the quantification of wtp53 protein expression is made by the light emitted by luciferase. Only wtp53 can bind to the p53RE, which allow the distinction of yeast with wtp53 or mutp53. **D)** The yeast phenotypic assay is based on the measure of yeast cell growth. With this assay is possible to screen compounds that restore the wtp53 function to mutp53 based on the growth inhibitory of wtp53, not observed with mutp53.

Over the last years, our group has been interested in the development of yeast-based screening assays with pharmacological purpose by targeting different proteins (e.g. p53 family proteins, p53 endogenous regulators, caspases, protein kinase C isoforms, mutp53 forms [248] and BRCA1/2. Our group developed a yeast phenotypic assay based on the wtp53-induced growth inhibition in yeast, which is evaluated by monitoring yeast

cell growth. These findings supported subsequent studies, in which the inhibitory effect of MDMs on p53 functionality in yeast was validated [196, 249]. This yeast phenotypic assay has allowed the identification of several small molecules that disrupt the MDM-p53 interaction including pyranoxanthone 1 [196], oxazolopyrrolidone 3a [197], prenylchalcone 2 [198], OXAZ-1 [207] and DIMP53-1 [208] by their ability to restore p53-dependent growth inhibitory effect. The non-interference of mutp53 with yeast cell growth has been also used by our group to screening for small molecules that restore the wt-like p53 activity to mutp53, based on their ability to rescue the wtp53-dependent yeast growth inhibition [233]. Indeed, SLMP53-1 was one of these compounds, firstly identified in yeast based on its ability to re-establish the inhibitory growth effect of mutp53 R280K [233]. In fact, this yeast phenotypic assay has been used by our group as first-line screening assay to streamline the identification of potential reactivators, which molecular mechanism of action is thereafter confirmed in human tumour cell lines.

Aims

In previous work, our group identified the small molecule MANIO as potential reactivator of mutp53, using the yeast phenotypic assay. With the present project, it was intended to:

- Validate the molecular mechanism of action of MANIO in human tumour cells;
- Study the antitumour activity of MANIO in human tumour cells and in human xenograft mice models.

2. Material and Methods

2.1 Reagents

MANIO was synthesized by Professor Dr. Teresa Pinto e Melo (Departamento de Química da Universidade de Coimbra). PRIMA-1^{MET} and etoposide (ETOP) were purchase from Sigma-Aldrich (Sintra, Portugal). All compounds were dissolved in dimethyl sulfoxide (DMSO; Millipore).

2.2 Plasmids

The pcDNA3 plasmid expressing different p53 mutants (R282W, R248W, R280K, R273H, R273C, G245D, R175H, R248Q, Y220C, G245S) or the empty pcDN3 plasmid were amplified in *Escherichia coli* DH5α from Lucigen (Frilabo, Porto, Portugal) and extracted using the GenEluteTM HP Plasmid Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions.

2.3 Human cell lines and growth conditions

The human colon adenocarcinoma HCT116 cell line expressing wtp53 (HCT116 p53$^{+/+}$) and its p53-null isogenic derivative (HCT116 p53$^{-/-}$) were provided by Dr. B. Vogelstein (The Johns Hopkins Kimmel Cancer Center, Baltimore, Maryland, USA); hepatocellular carcinoma Huh-7 cell line express mutp53 Y220C was provided by Dr. M. Prudêncio (Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa); human breast adenocarcinoma MCF-7 and human large cell lung cancer H460 cell lines expressing wtp53, human breast adenocarcinoma MDA-MB-468 and human colon adenocarcinoma HT-29 cell lines expressing mutp53 R273H, rectum carcinoma SW837 cell line expressing mutp53 R48W, and p53-null human non-small cell lung cancer H1299 and human infiltrating ductal breast carcinoma HCC1937 cell lines were purchased from ATCC (LGC Standards S.L.U., Barcelona, Spain). All cell lines were cultured in RPMI-1640 with Ultra Glutamine medium from Lonza (VWR, Carnaxide, Portugal) supplemented with 10% fetal bovine serum (FBS) from Gibco (Alfagene, Carcavelos, Portugal) and maintained in a humidified incubator at 37ºC with 5% CO2 in air.

2.4 Sulforhodamine B assay

Tumour cell lines were seeded in 96-well plates at 5.0 × 10^3 density (for HCT116, MCF-7, H460, Huh7 and H1299), 7.5 × 10^3 density (for HT29, SW837 and HCC1937) and

1.0 x 10^4 density (for MDA-MB-468) and incubated for 24h. Cells were treated with serial dilutions (0.1875-150 µM) of MANIO for 48h and the solvent (DMSO 0.25%) was used as control. MANIO effect on cell proliferation was analysed by sulforhodamine B (SRB) assay to determine the IC_{50} (the concentration that causes 50% of growth inhibition) values. Briefly, for cell fixation was used 10% trichloroacetic acid from Scharlau (Sigma-Aldrich). Plates were thereafter stained with 0.4% SRB (Sigma-Aldrich) and washed with 1% acetic acid. To solubilize the bound dye 10 mM Tris Base was used and the absorbance was measured in a microplate reader at 510 nm (Biotek Instruments Inc., Synergy MX, USA).

2.5 Colony formation assay

HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ tumour cells were seeded in 6-well plates 5.0 × 10^2 cells/well density and incubated with MANIO (0.019, 0.038, 0.075, 0.15 and 0.3 µM) or DMSO, for 14 days. Colonies were thereafter fixed using 10% methanol and 10% acetic acid for 10 minutes and stained with 0.5% crystal violet from Sigma-Aldrich for 15 minutes in 1:1 methanol/H_2O. Colonies containing more than 20 cells were counted.

2.6 Cell cycle and apoptosis

For cell cycle analysis, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ tumour cells were plated in 6-well plates at 1.5 × 10^5 cells/well density and incubated for 24h. After treatment with two concentrations of MANIO (1 and 2.5 µM) or DMSO only (control) for 48h, cells were fixed in ice-cold 70% ethanol. Cells were incubated with RNase A (Sigma-Aldrich) at final concentration of 20 µg/mL for 15 min at 37ºC, and then incubated with 50 µg/mL propidium iodide (PI) from Fluka (Sigma-Aldrich) for 30 min. For apoptosis analysis, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ tumour cells were also plated in 6-well plates at 1.5 × 10^5 cells/well density and incubated for 24h. Cells were treated with two concentrations of MANIO (1 and 2.5 µM) or DMSO only (control) for 48 and 72h, and incubated with Annexin V:FITC apoptosis Detection Kit I from BD Biosciences (Enzifarma, Porto, Portugal), according to the manufacturer's instructions. In both assays, cells were analysed by flow cytometry.

2.7 Western blot

HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ tumour cells were seeded in 6-well plates at 1.5 × 10^5 cells/well density for 24h. Cells were then treated with 0.5, 1 and 2.5 µM of MANIO or DMSO for 24, 48 and 72h. Whole proteins extracts were analysed by Western blot. Proteins (40 µg) were thereafter electrophoresed using a 10% SDS-PAGE, transferred to a Whatman nitrocellulose membrane from Protan (VWR, Carnaxide, Portugal) and in the end membranes were blocked with 5% milk. Membranes were probed with several primary antibodies overnight, followed by 2h incubation with a horseradish-peroxidase (HRP)-conjugated secondary antibody (Table 5). For loading control, membranes were stripped (Abcam protocol) and reprobed with an anti-GAPDH antibody (Table 5). For the detection of the signal was used the ECL Amersham kit from GE Healthcare (VWR) and ChemiDoc XRS+ from Bio-Rad. In this thesis, the Western blots presented are representative of three independent experiments.

Table 5 – Antibodies used in Western Blots.

Antigen	Final dilution	Secondary Antibody	Supplier
GADD45	1:100	Anti-Rabbit	Millipore
KILLER	1:200	Anti-Rabbit	Thermo Scientific
MDM2 (SMP14)	1:200	Anti-Mouse	Santa Cruz Biotechnology
PUMA (B-6)	1:50	Anti-Mouse	
p21 (C-19)	1:100	Anti-Rabbit	
p53 (DO-1)	1:5000	Anti-Mouse	
BCL2 (C-2)	1:100	Anti-Mouse	
PARP (C2-10)	1:1500	Anti-Mouse	
GAPDH (6C5)	1:10000	Anti-Mouse	
Anti-mouse HRP-conjugated	1:5000		
Anti-rabbit HRP-conjugated	1:5000		

2.8 Transient Transfection assay

The growth inhibitory effect of MANIO in mutp53 forms were evaluated through the exogenous expression of mutp53 in p53-null H1299 tumour cells. Tumour cells were transfected at 7.5×10^3 cells/well density with pcDNA3 plasmid, expressing different p53 mutants (R282W, R248W, R280K, R273H, R273C, G245D, R175H, R248Q, Y220C, G245S) or with the empty pcDNA3 plasmid, using ScreenFect®A from InCella (GRiSP) according to the manufacturer's instructions. The growth inhibitory effect was evaluated by SRB assay for 48h treatment with serial dilutions (3.13 – 50 µM) of MANIO.

2.9 In vitro migration assay

Cell migration was evaluated using the Wound Healing Scratch assay, as described in [250]. Briefly, HCT116 $p53^{+/+}$ tumour cells were seeded in 6-well plates at 5×10^5 cells/well density for 24h. After creating a wound in the middle of the well using a sterile 200µL tip, cells were treated with the 0.375 µM of MANIO (or DMSO only). Thereafter, cells were photographed using the Moticam 5.0MP camera with Motic's AE2000 inverted microscope with 100x magnification at different time-points of treatment until complete closure of the wound.

2.10 Comet assay

DNA damage was measured by comet assay in HCT116 $p53^{+/+}$ tumour cells. HCT116 tumour cells were seeded in 6-well plates at 1.5×10^5 cells/well for 24h, with subsequent treatment with 50 µM ETOP (as positive control), 1 µM and 2 µM MANIO or DMSO for 48h. The OxiSelect Comet Assay kit (Cell Biolabs, MEDITECNO, Carcavelos, Portugal) was used according to the manufacturer's instructions, with TBE (Tris/borate/EDTA) for electrophoresis. Cells were photographed (Nikon DS-5Mc camera and Nikon Eclipse E400 fluorescence microscope) and images processed using a Nikon ACT-2u software (Izasa). The quantification of comet-positive cells corresponds to cells containing more than 5% of DNA in the tail (tail moment corresponds to the product of the tail length and the percentage of DNA in the tail), assessed by Open Comet/ImageJ.

2.11 *In vivo* antitumour assay

Animal experiments were conducted according to the European Council Directive 2010/63/EU on Animal Care and to the National Authorities. Swiss nude mice (Charles-River Laboratories) were housed in individual ventilated cages under pathogen free conditions. Xenograft tumour assays were performed with HCT116 $p53^{+/+}$ tumour cells. Briefly, 1 x 10^6 HCT116 $p53^{+/+}$ cells (in PBS) were inoculated subcutaneously in the dorsal flank of mice. Tumour dimensions were evaluated by calliper measurement, with subsequent calculation of their volume [tumour volume = (L x W^2)/2]; L and W represent the longest and shortest axis of the tumour, respectively]. Treatment started ~14 days after the inoculation, when tumours reached approximately 100 mm^3 volume the grafts. Mice were treated twice a week for two weeks with 100 mg•kg^{-1} MANIO or vehicle by intraperitoneal injection. Throughout the treatment, tumour volumes and body weights were monitored twice a week as well. At the end of the antitumour assay, when tumours reached 1500 mm^3 or if the animals presented any signs of morbidity, animals were sacrificed by cervical dislocation and tumours were removed.Each group (control, treated) was comprised of six animals.

2.12 Flow cytometric data acquisition and analysis

To flow cytometric data acquisition was used the AccuriTM C6 flow cytometer (BD Biosciences). For the cell cycle phases identification and quantification, the FlowJo software was used.

2.13 Statistical analysis

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

3. Results

3.1 MANIO has a p53-dependent tumour growth inhibitory effect through induction of cell cycle arrest and apoptosis

In previous work, our group identified the small-molecule MANIO (patent request, UPIN – University of Porto) as a potential activator of wtp53 and reactivator of mutp53 forms using the yeast phenotypic assay developed by our group [248]. In the present thesis, MANIO was tested in human tumour cells in order to validate the molecular mechanism of action and to study its antitumour activity.

The activity of MANIO was firstly evaluated by SRB assay, after 48h treatment, in human colon, breast, lung and hepatocellular carcinoma cell lines expressing p53 (wt or mutant forms) and without p53 expression. The selectivity of MANIO to the p53 pathway was evidenced by its anti-proliferative effect in these different human tumour cell lines (Figure 9). The IC_{50} values of MANIO was much lower in tumour cells expressing wtp53 (0.2-0.97 µM) and mutp53 forms (0.2-3.2 µM) when compared to p53-null tumour cells (27.0-48.25 µM) (Figure 9).

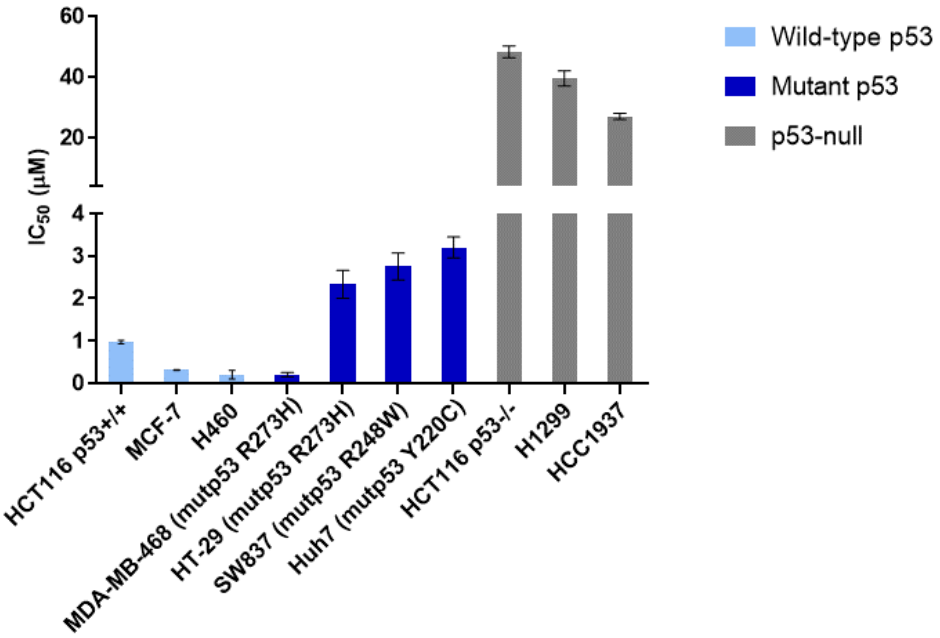

Figure 9 - Growth inhibitory effect of MANIO in human tumour cell lines. IC_{50} values were determined by the SRB assay, after 48h treatment, with MANIO. Data are mean ± SEM of 4-5 independent experiments.

In fact, the IC_{50} values of MANIO in HCT116 $p53^{+/+}$ cells (0.97 ± 0.04 µM) was 49-fold lower than that obtained in HCT116 $p53^{-/-}$ cells (48.25 ± 1.97 µM) (Figure 10A). Altogether, these results supported a p53-dependent antitumor activity of MANIO.

Using the same experimental conditions, the activity of MANIO was also compared to PRIMA-1^{MET} (a well-known reactivator of mutp53 in clinical trials [251]) (Figure 10B). Interestingly, contrary to MANIO, PRIMA-1^{MET} presented a p53-independent antitumor activity as demonstrated by the IC_{50} values obtained in $p53^{+/+}$ and $p53^{-/-}$ (19.50 vs. 6.57 µM, respectively) HCT116 tumour cells. The IC_{50} values also revealed a higher potency of MANIO in p53-expressing tumour cells compared to PRIMA-1^{MET}.

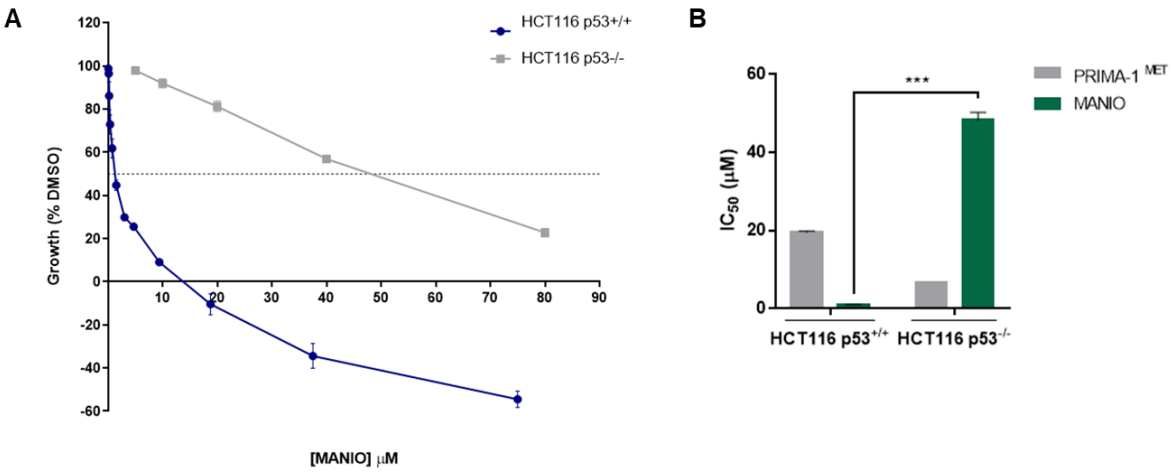

Figure 10 – Growth inhibitory activity of MANIO and PRIMA-1^{MET} in p53-expressing and p53-null HCT116 tumour cell lines. A) Concentration-response curves for the growth inhibitory effect of MANIO in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ tumour cells. The growth was determined by the SRB assay after 48h treatment with MANIO. Data are mean ± SEM (n=4-5). **B)** IC_{50} values of MANIO and PRIMA-1^{MET} in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ tumour cells, determined by the SRB assay after 48h treatment with MANIO and PRIMA-1^{MET}. Data are mean ± SEM (n=3-5; values significantly different: ***p<0.001 unpaired Student's *t*-test).

This p53-dependent growth inhibitory effect of MANIO in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells was further confirmed by colony formation assay. Also in this assay, MANIO reduced the colony formation ability of wtp53-expressing HCT116 cells, but not of p53-null HCT116 cells (Figure 11). Indeed, at the highest concentration tested (0.3 µM), MANIO reduced the colony formation ability of HCT116 $p53^{+/+}$ by 98.5%, compared to DMSO only (Figure

11). At the lowest concentration tested (0.019 µM), the compound was still capable to reduce the colony formation ability of HCT116 $p53^{+/+}$ by 33.4% (Figure 11).

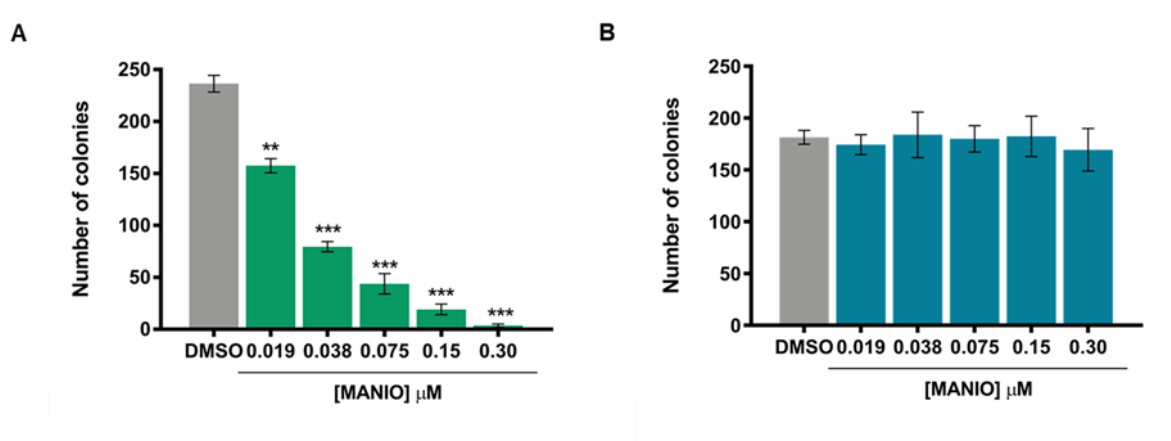

Figure 11 - Effect of MANIO on HCT116 $p53^{+/+}$ (A) and HCT116 $p53^{-/-}$ (B) cells by colony formation assay, after 14 days of MANIO treatment. Representative images of the colony forming assays. Data are mean ± SEM (n=4; values significantly different from DMSO: ***p*<0.01; ****p*<0.001; unpaired Student's *t*-test).

The p53-dependent tumour growth inhibitory effect of MANIO was associated with G2/M-phase cell cycle arrest (Figure 12A) and apoptosis (Figure 12B) in $p53^{+/+}$, but not in $p53^{-/-}$, HCT116 cells. In fact, in HCT116 $p53^{+/+}$ cells, the G2/M-phase cell cycle arrest was observed at 1 and 2.5 µM of MANIO, after 48h treatment (Figure 12A). Consistently, in HCT116 $p53^{+/+}$ cells, 1 and 2.5 µM MANIO presented a 2-fold and 4-fold increase of apoptosis at 48h and 72h treatment, respectively, compared to solvent (Figure 12B). Moreover, reinforcing the induction of a p53-dependent apoptotic cell death by MANIO, it was observed the promotion of PARP cleavage at 1 µM of compound in $p53^{+/+}$, but not in $p53^{-/-}$, HCT116 cells (Figure 12C).

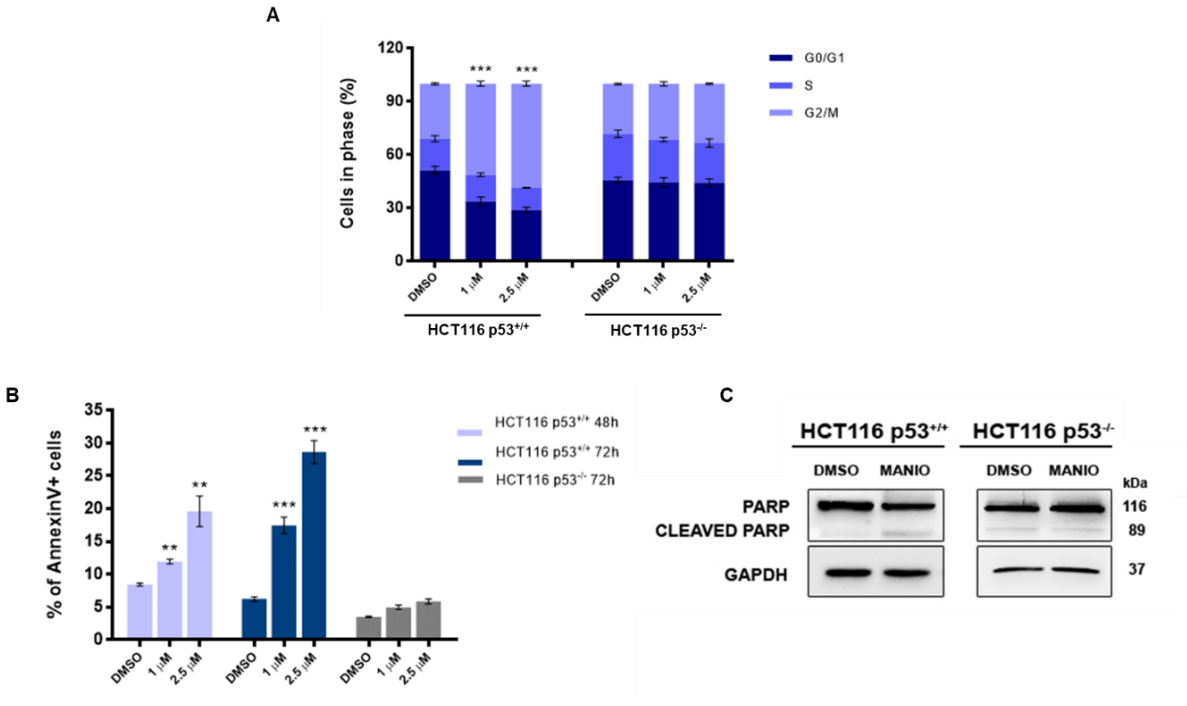

Figure 12 - MANIO-induced tumour growth inhibitory effect is associated with cell cycle arrest and apoptosis in p53$^{+/+}$, but not in p53$^{-/-}$, HCT116 cells. (A) Effect of MANIO on cell cycle progression of p53$^{+/+}$ and p53$^{-/-}$ HCT116 cells after 48h treatment. Data are mean ± SEM (n=3; values significantly different from DMSO: ***p<0.001; unpaired Student's t-test). **(B)** Effect of MANIO on apoptosis of p53$^{+/+}$ and p53$^{-/-}$ HCT116 cells, after 48 and 72h treatment. Data are mean ± SEM (n=3; values significantly different from DMSO: **p<0.01; ***p<0.001; unpaired Student's t-test). **(C)** Western blot analysis of cleaved PARP, after 72h treatment, with 1 μM MANIO in p53$^{+/+}$ and p53$^{-/-}$ HCT116 cells. Immunoblots represent one of three independent experiments. GAPDH was used as a loading control.

Overall, these results corroborate the p53-dependent tumour growth inhibitory effect of MANIO through the induction of cell cycle arrest and apoptosis.

3.2 MANIO increases the protein levels of p53 and modulates p53 transcriptional targets in a p53-dependent manner

The effect of MANIO on p53 activity was explored by analysis of the expression levels of p53 and of its transcriptional targets involved in cell cycle and apoptosis, in p53$^{+/+}$ and p53$^{-/-}$ HCT116 cells.

In HCT116 p53$^{+/+}$ cells, MANIO increased the p53 protein levels in a time- and concentration-dependent manner (Figure 13A).

Also in HCT116 $p53^{+/+}$ cells, 1 µM MANIO increased the expression levels of MDM2, p21, GADD45, PUMA and KILLER, and decreased the expression levels of BCL-2, after 48h or 72h treatment (Figure 13B). The increased expression levels of p21 and GADD45 corroborated the p53-dependent cell cycle arrest, and the modulation of the expression levels of PUMA, KILLER and BCL-2 indicated the involvement of p53 in the induction of apoptosis. Consistently, in HCT116 $p53^{-/-}$ cells, MANIO did not interfered with the expression levels of these p53 transcriptional targets, further supporting the p53-dependent activity of MANIO (Figure 13B).

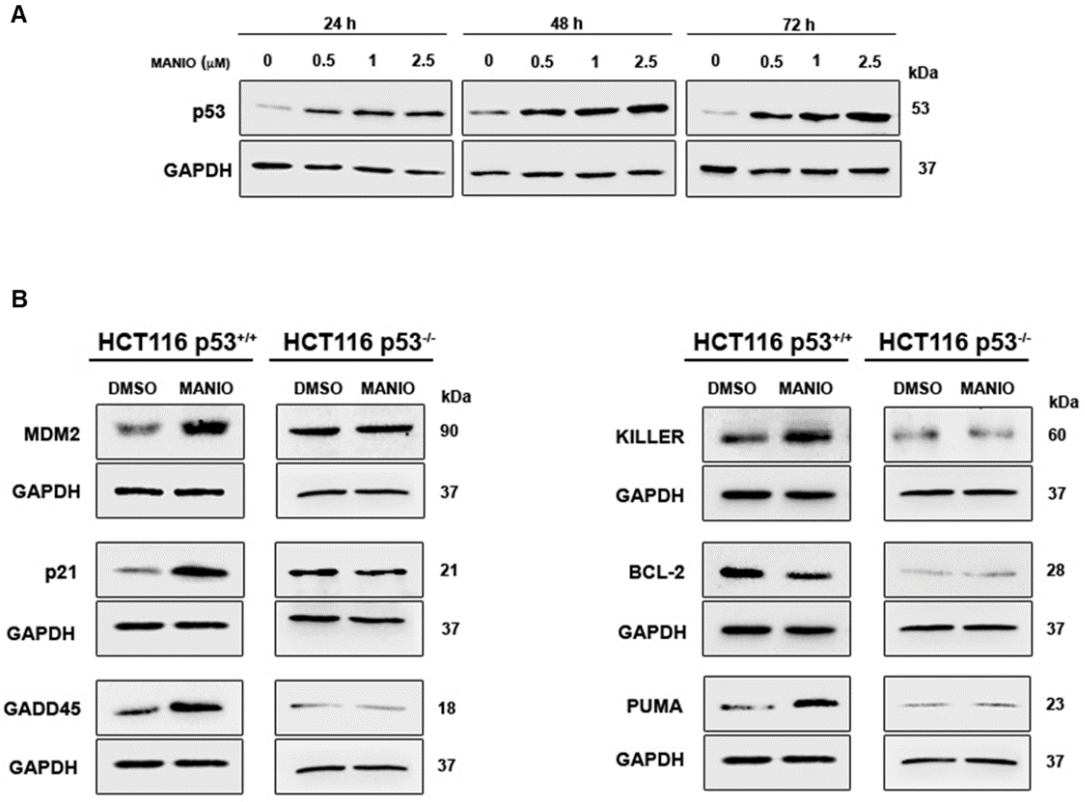

Figure 13 - MANIO increases the protein levels of p53 and modulates the expression of p53 transcriptional targets in $p53^{+/+}$, but not in $p53^{-/-}$ HCT116 cells. Results correspond to: **(A)** 24, 48, and 72h treatment with MANIO for p53; **(B)** 48h (for MDM2, p21, and GADD45) and 72h (for BCL-2, KILLER, and PUMA) treatment with 1µM MANIO. Immunoblots represent one of three independent experiments. GAPDH was used as a loading control.

Altogether, these results showed that MANIO induced p53 activation and stabilization, and interfered with p53 transcriptional activity through regulation of the expression levels of p53 target genes involved in cell cycle arrest and apoptosis.

3.3 MANIO potentially reactivates distinct mutp53 forms

To investigate the ability of MANIO to reactivate mutp53 in human tumour cells, several high prevalent human mutp53 forms (either DNA contact or structural missense mutations) were ectopically expressed in p53-null H1299 tumour cells. The growth inhibitory activity of MANIO in this panel of mutations was thereafter evaluated by SRB assay, after 48h treatment. Notably, with the exception of the structural mutp53 Y220C and G245S, the IC_{50} values of MANIO in mutp53-expressing H1299 cells were, in general, significantly lower compared to cells transfected with the empty vector (Figure 14). The R248W and R248Q, both with a missense mutation in the same codon, presented the highest reactivation levels compared to the empty vector (Figure 14). Of note that the IC_{50} value of non-transfected H1299 cells and transfected cells with the empty vector presented similar IC_{50} values of 39.5 ± 2.5 µM, confirming the non-interference of the transfection method with the activity of MANIO (Figure 14). Altogether, these results support the ability of MANIO to reactivate a broad panel of mutp53 forms in human tumour cells.

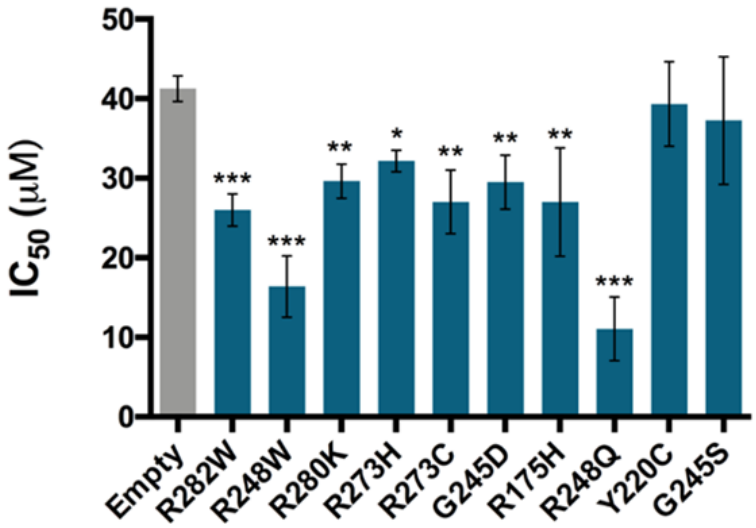

Figure 14 - Growth inhibitory effect of MANIO in mutp53 ectopically expressed in H1299 cells. H1299 cells were transfected with pcDNA3 plasmid expressing different p53 mutants (R282W, R248W, R280K, R273H, R273C, G245D, R175H, R248Q, Y220C, G245S), or with the empty pcDNA3 plasmid, using

ScreenFect® A. The growth inhibitory effect was evaluated by SRB for 48h treatment with MANIO. Data are mean ± SEM (n=3-10; values significantly different from pcDNA3-Empty: **p*<0.05 ***p*<0.01; ****p*<0.001 unpaired Student's *t*-test).

3.4 MANIO prevents *in vitro* wtp53-expressing tumour cell migration

The effect of MANIO in HCT116 $p53^{+/+}$ cells migration was evaluated by the wound healing assay. The results obtained showed that, at the GI_{30} (0.375 µM) concentration, MANIO visibly reduced the *in vitro* migration of HCT116 $p53^{+/+}$ cells, comparing to DMSO only, after 16h and 24h treatment (Figure 15). These results indicated that MANIO reduced the *in vitro* migration of tumour cells expressing wtp53.

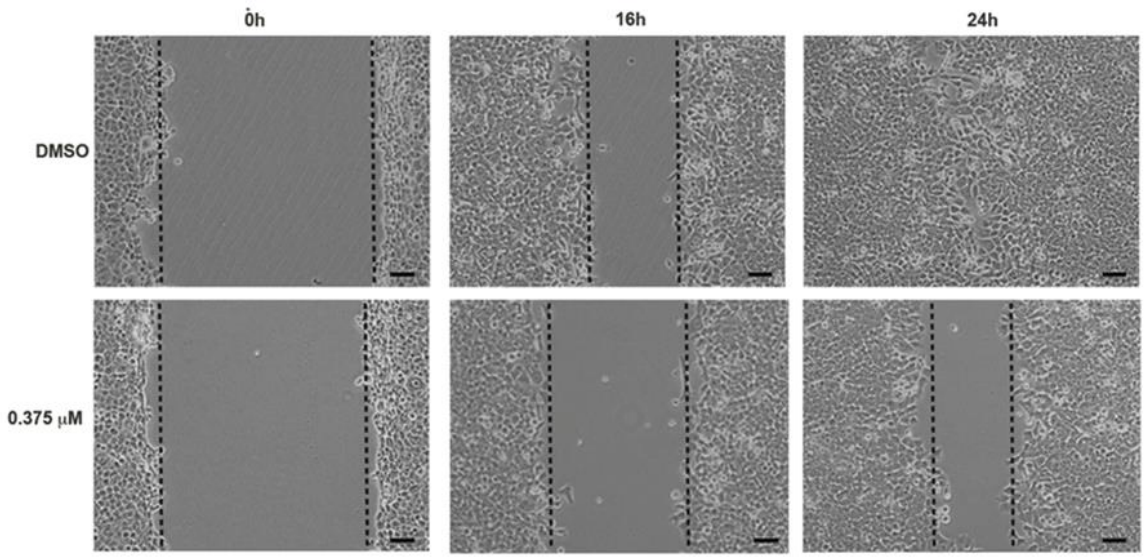

Figure 15 - MANIO prevents *in vitro* tumour cell migration of HCT116 $p53^{+/+}$ cells. Confluent cells treated with 0.375 µM MANIO, or DMSO only were observed at different time-points (0h, 16h and 24h) in the wound healing assay (scale bar=50 µm and magnification=100×).

3.5 MANIO is non-genotoxic in human tumour cells

The comet assay was performed to evaluate the genotoxicity of MANIO in HCT116 $p53^{+/+}$ tumour cells. The results showed that, unlike the positive control (ETOP), 1 and 2.5 µM MANIO did not increase the percentage of comet-positive cells when compared to DMSO (negative control) (Figure 16A and 16B). These results support that MANIO is a non-genotoxic compound in human tumour cells.

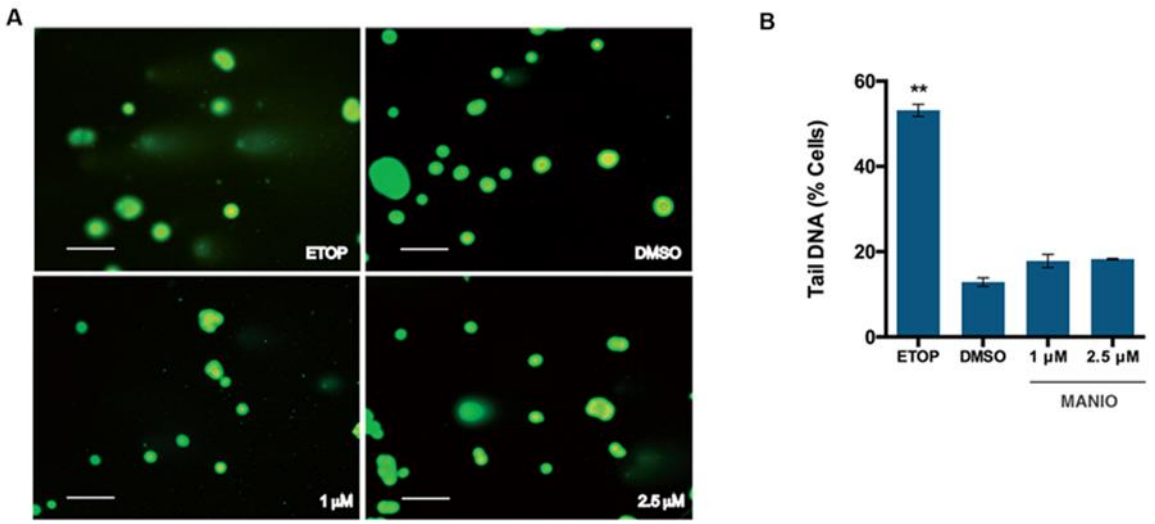

Figure 16 - MANIO is non-genotoxic in HCT116 p53$^{+/+}$ cells. (A) DNA damage was measured by comet assay in HCT116 p53$^{+/+}$ cells treated with 50 μM ETOP (positive control), 1 and 2.5 μM MANIO or DMSO only. Scale bar = 20 μm; Magnification = 100x. **(B)** Quantification of comet-positive cells (containing more than 5% of DNA in the tail; assessed by OPEN COMET/IMAGEJ); 100 cells were analysed in each group (n=3; values significantly different from DMSO: ***p*<0.01; unpaired Student's *t*-test).

3.6 MANIO has *in vivo* antitumour activity with no apparent secondary toxicity

The *in vivo* antitumour activity of MANIO was evaluated in human tumour xenograft mice models of HCT116 p53$^{+/+}$ cells. The results showed that four intraperitoneal administrations of 100 mg/kg MANIO significantly inhibited the tumour growth, particularly tumour volume (Figure 17A) and tumour weight (Figure 17B), when compared to the vehicle. Moreover, no significant loss of body weight was observed in treated mice, comparing to control, showing no apparent secondary toxicity induced by MANIO, throughout the experimental period (Figure 17C). These results validated the antitumor activity of MANIO, not associated with secondary toxic side effects.

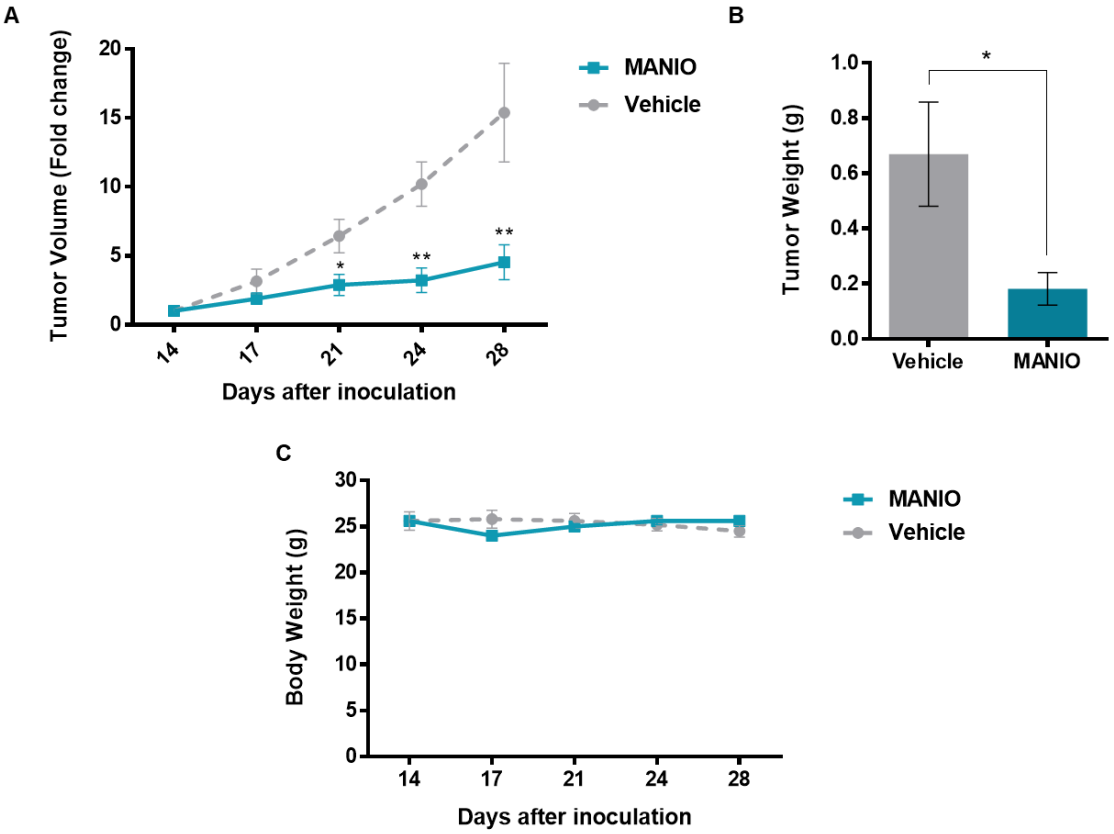

Figure 17 - MANIO has *in vivo* antitumor activity. (A) Tumour volume after swiss nude mice carrying HCT116p53$^{+/+}$ xenografts were treated with 100 mg/kg MANIO or vehicle (control); values significantly different from control mice (*p<0.05, **p<0.01; unpaired Student's t-test). **(B)** Tumour weight after MANIO or vehicle (control) treatment; data are mean ± SEM (*p<0.05; unpaired Student's t-test). **(C)** Mice body weight during MANIO or vehicle (control) treatment; no significant differences between control and MANIO-treated mice (p>0.05; unpaired Student's t-test). Each group (control and treated) was comprised of six animals.

4. Discussion and Final conclusions

The loss of the normal activity of p53 causes several cellular dysfunctionalities, either in neoplastic and non-neoplastic diseases [252]. Particularly in cancer, mutations in the *TP53* gene lead to the occurrence of therapeutic resistances and more aggressive tumour phenotypes reflected in poor prognosis [253, 254]. The restoration of the wt-like p53 function to mutp53 is considered a promising strategy to achieve improved clinical outcomes [174, 255]. Although several groups have already identified numerous compounds able to restore the wt-like activity to mutp53 [197, 199, 230, 231], only few have entered into clinical trials [228, 231] mainly because of the induction of toxicity and the incapacity to reactivate a broad panel of mutp53 forms.

In this thesis, it is reported the mechanism of action and the antitumour activity of MANIO, identified as a new potent reactivator of mutp53. In previous work, the small molecule MANIO revealed to be a potential reactivator of mutp53 in yeast [248]. Herein, the mechanism of action was validated in human tumour cells. In fact, the anti-proliferative activity of MANIO, contrary to PRIMA-1^{MET}, decreased significantly in several wtp53- and mutp53-expressing tumour cell lines, when compared to p53-null tumour cells. This p53-dependent growth inhibitory effect of MANIO occurs through induction of a G2/M-phase cell cycle arrest and apoptosis. Also, the induction of PARP cleavage, upon caspase-3 activation at the end of the caspase cascade, reinforces the induction of apoptosis [256, 257]. Moreover, the growth inhibitory effect in wtp53-expressing tumour cells by MANIO is associated with p53 stabilization and activation, as well as modulation of transcriptional targets of p53 involved in cell cycle and apoptosis. Consistently, MANIO up-regulates p21 and GADD45 expression levels, which conducts to the inhibition of the Cyclin B/Cdc2 complex, with subsequent induction of G2/M-phase cell cycle arrest [72, 73]. Likewise, MANIO up-regulates the expression levels of the pro-apoptotic proteins PUMA and KILLER/DR5, and down-regulates the expression levels of the anti-apoptotic Bcl-2 protein [85, 90, 94].

The ability of MANIO to reactivate either DNA contact or structural high prevalent human mutp53 forms was also confirmed. The lack of reactivation of mutp53 Y220C and G245S may be due to structural modifications on p53, blocking the binding of the compound to the protein. Despite the ability to activate a large panel of mutp53 forms, the compound demonstrated to have some selectivity for the 248 residue, since the highest reactivation levels were observed for mutp53 R248W and R248Q. These two DNA contact mutations acquire GOF activities, which were verified in several humanized mice model studies. Particularly, mutp53 R248Q accelerates tumour onset [258] and mutp53 R248W interacts with new partners such as MRE11 protein to block the ATM-dependent DNA repair, leading to poor prognosis [170]. In addition, these two mutations are highly

prevalent in both Li Fraumeni and somatic tumours, according to the *TP53* mutation database [145]. The reactivation of these two mutations by MANIO has high clinical relevance. Firstly, the reactivation of these mutations in cancer patients can improve the prognosis of these patients, through the reversion of the GOF activity of these mutations. Secondly, chemoprevention is an important strategy in patients with the Li-Fraumeni syndrome, since these patients present a high risk of developing cancer [reviewed in [259]]. Therefore, MANIO reactivation of mutp53 R248W and R248Q, that are highly prevalent in Li-Fraumeni patients, can also be used as a chemoprevention strategy in these patients, reducing the risk of developing cancer.

Furthermore, the activation of tumour cell invasion and metastases is one of the hallmarks of cancer that still remains a major cause of mortality in cancer [8]. It is well-stablished the key role of p53 in preventing tumour migration, and that mutp53, particularly its GOF activity, is associated with more invasive and metastatic tumours [reviewed in [260]]. Herein, low doses of MANIO has the ability to reduce the *in vitro* migration of wtp53-expressing tumour cells, reinforcing the reactivation of wtp53 and the *in vitro* antitumour potential of the compound.

One of the highest problems in oncology is the drug toxicity, restricting the therapeutic options specially in metastatic cancer patients [reviewed in [261]]. MANIO shows to be non-genotoxic in human tumour cells. These *in vitro* results are corroborated by the no significant loss of body weight in MANIO-treated mice, demonstrating that MANIO has no apparent toxic side effects in normal tissues. Besides this, the antitumor activity of MANIO is also confirmed in xenograft mice models of human wtp53-expressing colon tumour cells. Further studies are required to elucidate the *in vivo* activity of the compound in p53-null and mutp53-expressing tumours.

In conclusion, this thesis reports the identification of a new reactivator of wtp53 and mutp53, the compound MANIO. Besides its potent antitumour activity, unlike others p53 reactivators already identified, MANIO is able to reactivate a broad panel of mutp53 forms. This new potential anticancer drug candidate might have promising clinical applications, particularly by improving the prognosis of cancer patients with an impaired p53 pathway.

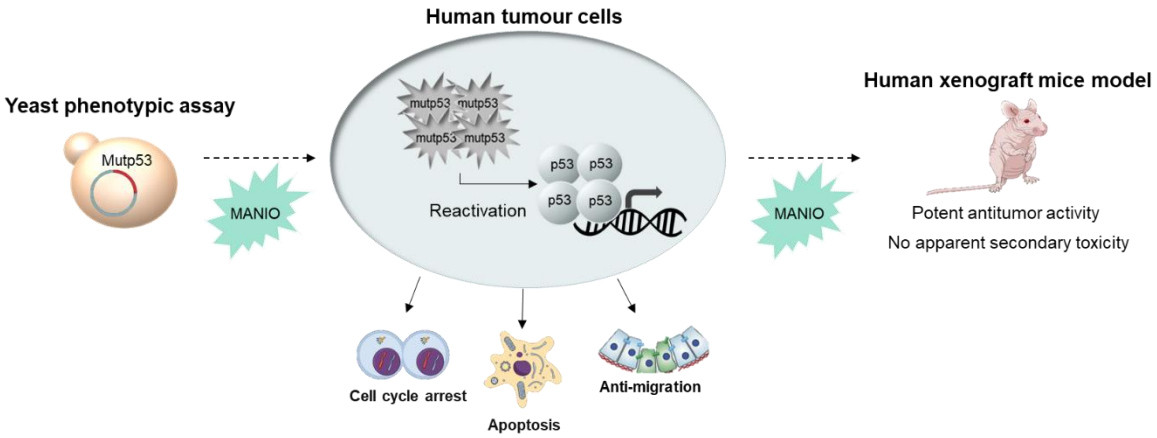

Figure 18 - Drug approach for the identification of MANIO. Firstly, MANIO was identified in the yeast phenotypic assay, as a potential mutp53 reactivator, in previous work. Herein, the molecular mechanism of action of the compound was validated initially *in vitro*, in which the p53-dependent tumour growth inhibitory effect was associated with cell cycle arrest, and apoptosis; anti-migration properties of the compound were also shown. Thereafter, the antitumor activity and absence of toxic side effects were validated *in vivo* using a xenograft mice model.

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