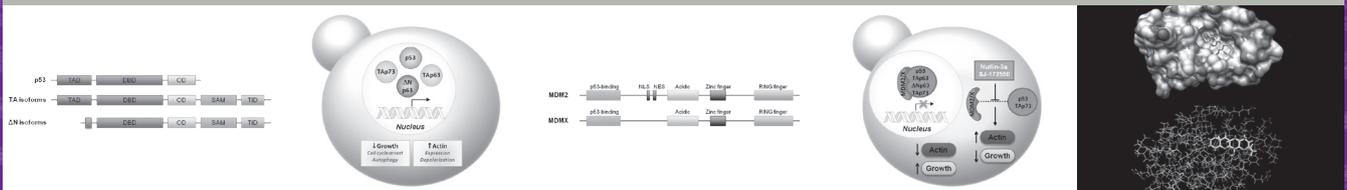




DEVELOPMENT OF YEAST-BASED ASSAYS TO STUDY P53 FAMILY PROTEINS: IDENTIFICATION OF NEW SMALL MOLECULE MODULATORS



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TESE APRESENTADA PARA ADMISSÃO A PROVAS DE DOUTORAMENTO
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**DEVELOPMENT OF YEAST-BASED ASSAYS TO STUDY P53
FAMILY PROTEINS: IDENTIFICATION OF NEW SMALL
MOLECULE MODULATORS**

Tese do 3º ciclo de estudos conducente ao grau de doutoramento em Ciências Farmacêuticas, ramo Microbiologia.

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Co-Orientadora Doutora Clara Isabel Ferreira Pereira

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Thesis cover figures:

- 1- Structure of p53 family proteins [Adapted from (Wei et al., 2012)];
- 2- Yeast p53 family assay;
- 3- MDM2 and MDMX: schematic representation of major domains [Adapted from (Lenos and Jochemsen, 2011)];
- 4- Yeast p53 family-MDMs assay;
- 5- Pyranoxanthone **1** in the binding site of MDM2.

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence”.

Louis Pasteur

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Abstract

The p53 family consists of three members, p53, p63 and p73 with a central role in the control of cell differentiation, proliferation and death, among others. This family of proteins is therefore considered a key therapeutic target in cancer. Although the intense research around these proteins, the biological and pharmacological profile of several p53 family members remains far from being completely understood, particularly due to the high complexity of the p53 pathway in mammalian cells. The function of these proteins is negatively regulated by interactors, such as murine double minute (MDM)2 and MDMX. However, contrary to p53 and p73, the regulation of p63 by MDM2/MDMX is still controversial. In spite of this, the inhibition of these interactions with MDM2/MDMX represents a promising therapeutic strategy to reactivate p53 family proteins in tumours. Despite the huge number of inhibitors of the p53-MDM2 interaction discovered in the last few years, only few inhibitors of the p53-MDMX interaction and dual inhibitors of the p53-MDM2/MDMX interaction have been reported. Similarly, inhibitors of the p63 and 73 interaction with MDM2/MDMX remain mostly unknown.

In the present thesis, the yeast *Saccharomyces cerevisiae* was used as a cell model for an independent analysis of human p53, p63 (full length and truncated forms) and p73 (full length) in a simplified eukaryotic cell system. Using this approach, several aspects of the biology of p53 family proteins were studied, particularly their regulation by MDM2/MDMX. Several similarities among the activities of the different p53 family proteins expressed in yeast were observed. In fact, all of them induced growth inhibition associated with S-phase cell cycle arrest and an autophagic cell death, reactive oxygen species generation, increase of actin depolarization and protein expression levels. Despite this, different sensitivities to the autophagosome pathway, to the inhibitory effect of MDM2 and MDMX, and to small molecule modulators of their interactions with MDM2/MDMX clearly emerged.

The applicability of the yeast cell models developed for p53 family proteins, alone and combined with MDM2/MDMX, as a drug screening approach was also validated in this work with the discovery of promising modulators of the activity of these proteins. Particularly, with the developed yeast p53-MDM2 screening assay, the pyranoxanthone **1** (LEM1) was identified as the first small molecule inhibitor of the p53-MDM2 interaction with a xanthone scaffold. In fact, with the validation of the molecular mechanism of action of this compound in human tumour cells, a proof of concept was therefore provided for the effectiveness of this cell system for the screening of inhibitors of the p53 interaction with its negative regulators. Thereafter, using the same yeast assay, α -mangostin and gambogic acid, were identified as potential inhibitors of the p53-MDM2 interaction. This

study contributed to the elucidation of the molecular mechanism of action of these two xanthenes well-known by their potent antitumor properties. Moreover, with the developed yeast p73-MDM2 interaction assay, the xanthone LEM2 was identified as an activator of the p73 pathway through potential MDM2 inhibition.

An additional outcome of this thesis was the identification of *ACT1* as a putative p53 family proteins target gene in yeast. This study may lead to the development of a simplified yeast p53 family proteins transactivation assay for the analysis of the p53 family proteins transcriptional activity in alternative to the more complex artificial yeast transactivation reporter assays traditionally used.

In conclusion, the data emerged from this thesis confirm the enormous potential of the yeast cell model to the study of human disease-associated proteins. Several relevant insights about the biology and potential druggability of these human proteins are revealed for the first time in yeast. Additionally, new yeast target-directed approaches are provided for future studies of the p53 family network, particularly for the identification of new regulators of the activity of p53 family proteins. These assays have proved to be highly effective as a first-line approach, before studies in more complex cell systems. It is therefore anticipated that, for the near future, the use of these cell systems holds great promise in the discovery of novel anticancer agents with therapeutic applicability.

Keywords: Cancer; MDM2/MDMX; p53 family proteins; Target-directed screening assays; Yeast

Resumo

A família da p53 consiste em três membros, p53, p63 e p73, os quais desempenham um papel central no controlo da diferenciação, proliferação e morte celular, entre outros processos. Neste sentido, esta família de proteínas é considerada um alvo terapêutico chave no cancro. Apesar da investigação intensiva de que têm sido alvo, o perfil biológico e farmacológico dos diversos membros da família da p53 está longe de ser completamente compreendido, principalmente devido à elevada complexidade das suas vias em células de mamífero. A função destas proteínas é modulada negativamente por reguladores endógenos, como as proteínas *murine double minute* (MDM)2 e MDMX. No entanto, contrariamente ao observado com p53 e p73, a regulação da p63 pelas proteínas MDM2/MDMX é ainda controversa. Apesar disto, a inibição das suas interações com MDM2/MDMX representa uma estratégia terapêutica promissora na reativação das proteínas da família da p53 em tumores. Apesar do número elevado de inibidores da interação p53-MDM2 descobertos nos últimos anos, poucos foram os inibidores da interação p53-MDMX e os inibidores duais das interações p53-MDM2/MDMX descritos até à presente data. De forma semelhante, inibidores da interação da p63 ou p73 com as proteínas MDM2/MDMX permanecem quase desconhecidos.

Na presente tese, a levedura *Saccharomyces cerevisiae* foi utilizada como modelo celular para uma análise das proteínas p53, p63 (forma completa e formas truncadas) e p73 (forma completa) expressas individualmente num sistema celular eucariótico simplificado. Usando esta abordagem, diversos aspetos da biologia das proteínas da família da p53 foram estudados, particularmente a sua regulação pelas proteínas MDM2/MDMX. Diversas similaridades entre as atividades das diferentes proteínas da família da p53 expressas em levedura foram observadas. De facto, todas elas induziram uma inibição do crescimento associada a uma retenção do ciclo celular na fase S e morte celular autofágica, geração de espécies reativas de oxigénio, aumento da despolarização e dos níveis de expressão da actina. Apesar disto, diferentes sensibilidades para a via do autofagossoma, para o efeito inibitório das proteínas MDM2 e MDMX e para os compostos moduladores das suas interações com as proteínas MDM2 e MDMX, foram observados no âmbito da presente tese.

A aplicabilidade dos modelos de levedura desenvolvidos para as proteínas da família da p53, expressas isoladamente ou combinadas com MDM2/MDMX, na pesquisa de novos fármacos, foi também validada neste trabalho com a descoberta de moduladores promissores da actividade destas proteínas. Em particular, com o ensaio de pesquisa de levedura desenvolvido para p53-MDM2, a piranoxantona **1** (LEM1) foi

identificada como o primeiro inibidor da interação p53-MDM2 com um núcleo xantônico. De facto, com a validação do mecanismo molecular de ação deste composto em células tumorais humanas, uma prova de conceito foi portanto obtida para a eficácia deste sistema celular na pesquisa de inibidores da interação da p53 com os seus reguladores negativos. Num trabalho subsequente, usando o mesmo ensaio de levedura, o α -mangostina e o ácido gambóxico foram identificados como potenciais inibidores da interação p53-MDM2. Este estudo contribuiu para a elucidação do mecanismo molecular de ação destas duas xantonas conhecidas pelas suas potentes propriedades anti-tumorais. Adicionalmente, com o ensaio de levedura desenvolvido para a interação p73-MDM2, a xantona LEM2 foi identificada como um ativador da via da p73 através de uma potencial inibição da proteína MDM2.

Com esta tese foi ainda possível identificar o gene *ACT1* como um potencial alvo das proteínas da família da p53 na levedura. Este estudo abriu caminho a um novo ensaio simplificado de transactivação das proteínas da família da p53 em levedura para a análise das suas atividades transcricionais. Este ensaio poderá ser utilizado em alternativa aos ensaios de transactivação tradicionais que têm por base um sistema de levedura artificial que utiliza um gene repórter.

Em conclusão, os dados obtidos nesta tese confirmam o enorme potencial do modelo de levedura no estudo de proteínas associadas a doenças humanas. Informações relevantes acerca da biologia destas proteínas, assim como do potencial desenvolvimento de seus moduladores farmacológicos, são reveladas pela primeira vez em levedura. Adicionalmente, novos ensaios de levedura direcionados para um determinado alvo são implementados, os quais poderão ser utilizados em estudos futuros das proteínas da família da p53, particularmente na identificação de novos reguladores das suas atividades. Estes ensaios provaram ser altamente eficientes quando utilizados numa fase inicial da pesquisa, antes dos tradicionais ensaios em sistemas celulares mais complexos. O uso destes sistemas celulares revela-se, desta forma, muito promissor na descoberta de novos agentes anticancerígenos com aplicabilidade terapêutica num futuro próximo.

Palavras-chave: Cancro; Ensaios de pesquisa direcionados para um alvo; Levedura; MDM2/MDMX; Proteínas da família da p53

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Abbreviations

ABP140	Actin binding protein 140
AIF	Apoptosis inducing factor
AMPK	Adenosine monophosphate activated protein kinase
Apaf-1	Apoptosis protease-activating factor 1
Bax	Bcl-2 associated X protein
Bak	Bcl-2-antagonist/killer
Bid	BH3 interacting-domain death agonist
CAK	CDK-activating kinase
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
CFU	Colony-forming unit
Cyt c	Cytochrome c
DAPK-1	Death associated protein kinase 1
DBD	DNA-binding domain
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
EndoG	Endonuclease G
FADD	Fas-associated protein with death domain
FASAY	Functional analysis of separated alleles
GADD45	Growth arrest and DNA-damage inducible protein
GFP	Green fluorescent protein
HTS	High-throughput screening
H₂O₂	Hydrogen peroxide
IAP	Inhibitor of apoptosis
MDM2	Murine double minute 2
MDMX/4	Murine double minute X/4
MOMP	Mitochondrial outer membrane permeabilization
mtCLIC	Mitochondrial chloride intracellular channel
mTOR	Molecular target of rapamycin
mt	Mutant
OD	Oligomerization domain
OD₆₀₀	Optical density at 600 nm
p53AIP1	p53-regulated apoptosis inducing protein 1
p53RE	p53 responsive element

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFT-α/μ	Pifthrin- α/μ
Pgk1p	Phosphoglycerate kinase
PI	Propidium iodide
pI	Isoelectric point
PUMA	p53 upregulated modulator of apoptosis
Q-PCR	Quantitative PCR
Rb	Retinoblastoma protein
RE	Response element
RLU	Relative light units
ROS	Reactive oxygen species
SAM	Sterile-alpha motif
SESN2	Sestrin 2
SCF	Skp, Cullin, F-box containing complex
SMAC	Second mitochondria-derived activator of caspases
SRB	Sulforhodamine B
TAD	Transactivation domain
TID	Transactivational inhibitory domain
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TSC2	Tuberous sclerosis protein 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
wt	Wild-type
Y2H	Yeas two-hybrid

Chapter 1

Introduction

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Cancer is among the leading causes of death in economically developed countries. Despite the improvement in the relative survival rates for many types of cancer, the cancer incidence is increasing as a result of population aging and cancer-associated lifestyle choices. According to the World Health Organization, in 2012, there were 14.1 million new cases of cancer, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis). In the same year, in Europe, 3.7 million new cases of cancer were diagnosed and 1.9 million people died with the disease (Bray et al., 2012; Ferley et al., 2012). These statistics data may justify why cancer has been the focus of an intense worldwide scientific research. In fact, during the last years, an enormous effort has been dedicated to the improvement of diagnostic techniques, accuracy of prognosis and cancer treatment [reviewed in (Hanahan and Weinberg, 2011)].

Carcinogenesis is a multistep process that involves dynamic changes in the genome. Usually, this process is associated with an abnormal cell growth and a defective cell death due to the oncogene activation and/or tumour suppressor gene inactivation. These genetic alterations drive the progressive transformation of normal cells into highly malignant derivatives [reviewed in (Hanahan and Weinberg, 2000)]. During this transformation process, normal cells acquire several capabilities, called hallmarks of cancer. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis [reviewed in (Hanahan and Weinberg, 2000)]. Recently, two emerging hallmarks were added to this list: the capability to reprogram the energy metabolism, in order to control the neoplastic transformation, and the capability of cancer cells to evade immunological destruction [reviewed in (Hanahan and Weinberg, 2011)].

Due to its central role in cell differentiation, proliferation and death, the p53 tumour suppressor protein has been a major therapeutic target in cancer [reviewed in (Lane et al., 2010)]. In fact, during the last years, a p53-targeted approach has yielded very promising therapeutic tools, particularly small molecules that target the p53-MDM2 interaction (e.g., Nutlin-3a) [reviewed in (Wade et al., 2013)]. However, the complexity of the p53 pathway has grown significantly, and we should no longer think of p53 as a 'lone warrior' in tumour suppression, mostly because p63 and p73 proteins are also implicated in cancer progression and metastasis [reviewed in (Candi et al., 2014)]. For these reasons, the development of innovative therapeutic approaches targeting not only p53, but also other p53 family members reveals to be a promising strategy to minimize the emergence of resistance and to achieve maximal therapeutic responses in cancer therapy.

1.1. THE P53 FAMILY PROTEINS

The p53 protein was discovered in 1979 as a protein that binds to the SV40 tumour-virus oncoprotein, the large T-antigen (Linzer and Levine, 1979; Lane and Crawford, 1979). At this time, p53 was considered an oncogene. However, this idea changed because the p53 initially discovered was in fact a mutant form of p53. Additionally, subsequent studies demonstrated that different types of tumours highly express a wild-type (wt) p53 form which, contrary to mutant p53, acts as a tumour suppressor protein [reviewed in (Lane and Levine, 2010)]. It was also found that p53 is a sequence-specific DNA-binding transcription factor, which controls the transcription of an assortment of genes involved in different cellular processes in order to prevent tumour formation [reviewed in (Lane and Levine, 2010)]. Due to its relevance as a tumour suppressor protein, p53 is also called “The Guardian of the Genome” (Lane, 1992).

Latter, two *TP53*-related genes were identified: *TP73* that encodes p73 (Kaghad et al., 1997) and *TP63* that encodes p63 (Yang et al., 1998). In spite of their structural homologies, the overlap in cellular functions between p53, p63 and p73 is limited. In fact, studies in knockout mice revealed that each protein of the p53 family has its unique functions [reviewed in (Murray-Zmijewski et al., 2006)]. p53-null mice are viable but die at an early age due to spontaneous cancers (Donehower et al., 1992). p73-null mice display neurological, pheromonal and inflammatory defects resulting in death within two months (Yang et al., 2000), while p63-null mice die at birth and exhibit growth abnormalities (Mills et al., 1999). The p53 family members also appear to have different functions in human biology. p53 is a well-established tumour suppressor and is one of the most frequently mutated proteins in sporadic cancers. *TP53* germline mutations are associated with the development of the cancer-prone Li-Fraumeni and Li-Fraumeni-like syndromes [reviewed in (Malkin, 2011)]. Conversely, p63 is critical for the correct development of ectodermal-derived tissues, and p63 germline mutations are associated with a subset of ectodermal dysplasia syndromes [reviewed in (Rinne et al., 2007)]. p73 contributes to neural and immune systems functions [reviewed in (Bourdon, 2007)] and, to date, no syndromes associated with p73 germline mutations have been identified. Contrary to *TP53*, mutations in *TP63* and *TP73* genes are rare. The role of these proteins in tumorigenesis is highly complex mainly due to the existence of several isoforms in a same cellular context. However, evidence emerged that both p63 and p73 exert a tumour suppression function in many human tumours [reviewed in (Candi et al., 2014)].

1.1.1. Structural organization

The *TP53*, *TP63* and *TP73* genes are located in different chromosomes, 17p13.1, 3q27-29 and 1p36.2-3, respectively. Each gene encodes proteins with a high degree of structural homology, sharing three domains that are essential for their functions: a transactivation domain (TAD), a DNA binding domain (DBD) and an oligomerization domain (OD). The highest homology is found in the DBD (Fig. 1) [reviewed in (Wei et al., 2012)].

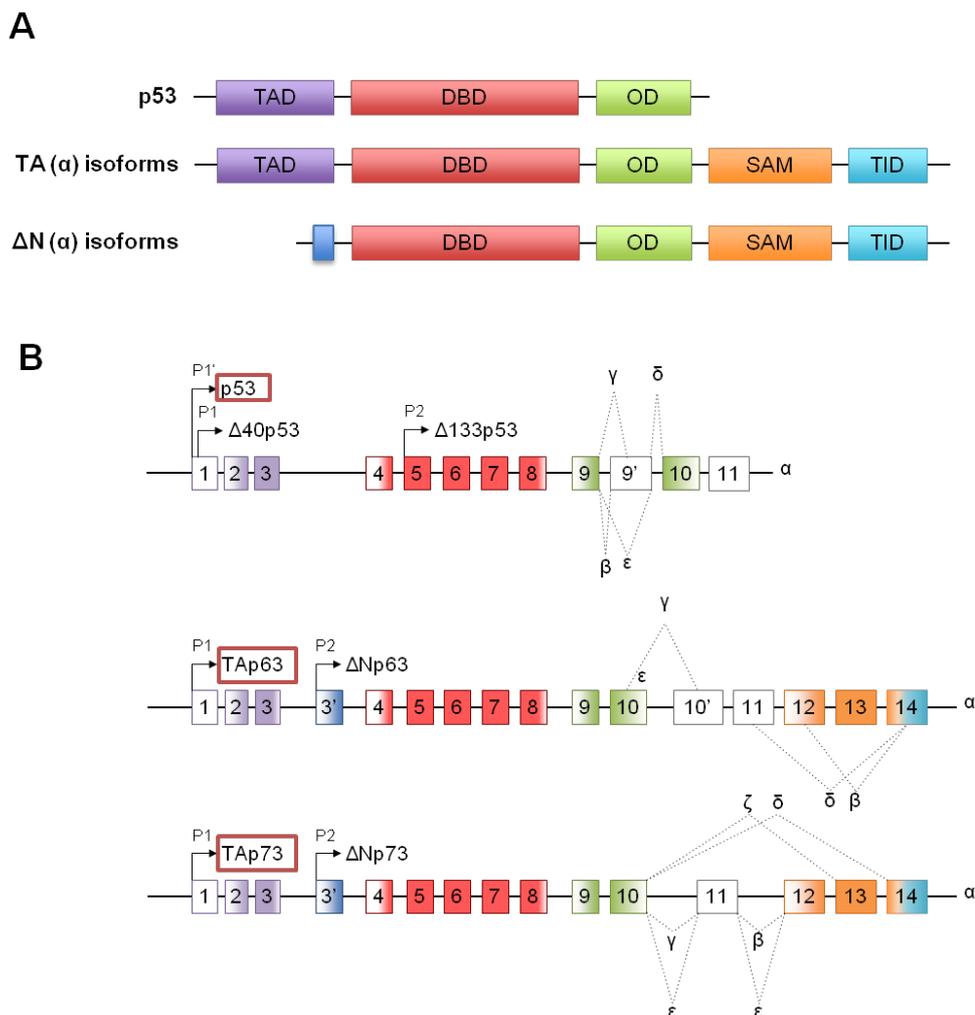


Figure 1. Structural organization of p53 family proteins. (A) Structure of p53 family proteins. p53, p63 and p73 consist of an amino-terminal transactivation domain (TAD), a central DNA binding domain (DBD) and a carboxy-terminal oligomerization domain (OD). α - Isoforms of p63 and p73 encode an additional Sterile Alpha Motif (SAM) domain followed by a transactivational inhibitory domain (TID). **(B) Architecture of human *TP53*, *TP63* and *TP73* genes.** Transcription of p53 family genes is controlled by two promoters, P1 and P2. *TP53* presents an additional promoter, P1'. N- or C- terminal alternative mRNA splicing leads to the generation of different

isoforms of each protein. Numbered boxes indicate exons. C-terminal splicing events for all p53 family members are indicated by dotted lines and Greek letter designation [Adapted from (Wei et al., 2012)].

Using different promoters (P1 and P2) and alternative mRNA splicing, *TP53*, *TP63* and *TP73* genes give rise to multiple protein isoforms with different functions (Fig. 1). In fact, while proteins resulting from the P1 promoter contain a N-terminal acidic TAD and are called TA proteins (TAp63 and TAp73), proteins resulting from the P2 promoter lack the entire N-terminal TAD and are called Δ N proteins (Δ Np63 and Δ Np73). Concerning the *TP53* gene, it includes three promoters: the P1' promoter that generates proteins containing the full TAD, the P1 promoter that gives rise to proteins lacking the first 40 amino acid residues (Δ 40p53; containing a N-terminal acidic TAD), and the P2 promoter that produces proteins lacking the first 133 amino acids (Δ 133p53; lacking all or part of the TAD) (Fig. 1) [reviewed in (Wei et al., 2012)].

Additional complexity is caused by alternative splicing at the C-terminal of p63 and p73 transcripts, generating a variety of TA and Δ N isoforms: eight splice variants for p53 (α , β , γ , δ , ϵ , ζ , Δ p53 and Δ E6), five for p63 (α , β , γ , δ and ϵ) and nine for p73 (α , β , γ , δ , ϵ , θ , ζ , η and η 1) (Fig. 1). Among the different generated isoforms, α , β and γ have been considered the most relevant [reviewed in (Wei et al., 2012)].

Moreover, contrary to p53, α isoforms of p63 and p73 have an additional Sterile Alpha Motif (SAM) domain in the C-terminal that is responsible for protein-protein interactions and transcriptional repression (Fig. 1) [reviewed in (Wei et al., 2012; Candi et al., 2014)]. Additionally, p63 α and p73 α have an additional domain designated as transactivational inhibitory domain (TID) (Fig. 1). The presence of this domain in p63 α and p73 α proteins reduces their transactivation activity through intra- or intermolecular associations with the TAD [reviewed in (Candi et al., 2014)]. Besides, the transactivation potential of TAp63 and TAp73 depend on the isoform. In fact, TAp63 γ and TAp73 β have been shown to be as potent as p53 regarding the induction of gene transcription and apoptosis [reviewed in (Wei et al., 2012)].

1.1.2. Biological functions

The p53 protein is a potent transcriptional regulator that binds to specific DNA sequences, the p53-responsive elements (p53REs), regulating an assortment of genes involved in major cellular pathways [reviewed in Riley et al., 2008)]. In unstressed cells,

p53 is maintained at low levels due to the presence of its main negative regulator murine double minute 2 (MDM2) that target it for proteasomal degradation. In response to different stress signals such as, DNA damage, hyperproliferative signals, hypoxia, oxidative stress, ribonucleotide depletion and nutrient starvation, p53 is displaced from MDM2 being stabilized and activated (Aylon and Oren, 2011; Bieging et al., 2014). Once activated, p53 accumulates in the nucleus and induces cell cycle arrest, DNA repair, senescence, apoptosis, and autophagy, among others [reviewed in (Ozaki and Nakagawara, 2011; Sui et al., 2011; Stegh 2012)]. Additionally, it is well established that p53 can exert its tumour suppressive function by transcription-independent mechanisms [reviewed in (Stegh 2012)].

Several functions initially attributed to p53 only are now shared by p63 and p73. Due to their structural homology with p53, particularly in the DBD, TAp63 and TAp73 are able to transactivate p53REs. Indeed, similarly to p53, in response to DNA damage, p63 and p73 induce cell cycle arrest, senescence and apoptosis [reviewed in (Khoury and Bourdon, 2010; Dötsch et al., 2010)]. Despite the important role of TAp63 and TAp73 in tumorigenesis, in unstressed cells, these proteins have unique biological functions, such as the regulation of differentiation and development [reviewed in (Dötsch et al., 2010)]. In contrast, it is known that the roles of Δ Np63 and Δ Np73 isoforms are multifaceted. In fact, Δ N isoforms can function as dominant-negative inhibitors of p53, TAp63 and TAp73 counterparts through promoter competition or hetero-complex formation. In promoter competition, Δ N isoforms compete with TA isoforms for their target gene promoters, preventing their transactivation function. In the hetero-complex formation mechanism, Δ N isoforms can inhibit TA isoforms by forming transcriptionally inactive hetero-oligomers with TA variants [reviewed in (Vilgelm et al., 2008; Wei et al., 2012)]. Additionally, a negative feedback loop is established between TA and Δ N isoforms. For instance, TA isoforms induce the transcription of Δ N isoforms by direct activation of the P2 promoter. In turn, Δ N isoforms inhibit TA isoforms [reviewed in (Dötsch et al., 2010; Wei et al., 2012)]. In fact, it was demonstrated that the expression levels of Δ Np73 are regulated by both p53 and p73 by direct activation of the P2 promoter of p73, creating an auto-regulatory feedback loop (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002). In addition, it was also reported that p53 induces Δ Np63 expression (Harmes et al., 2003). The effect of these interactions in a cellular context appears to be dependent on a balance between TA and Δ N isoforms. In fact, deregulation of the TA/ Δ N expression ratio may lead to tumour development [reviewed in (Wei et al., 2012)]. Besides their dominant negative effect on TA isoforms, it was demonstrated that Δ N isoforms are also transcriptionally active using their own transcriptional programs due to the existence of additional TA domains

[reviewed in (Wei et al., 2012)]. In fact, it was reported that ΔN isoforms can bind to p53REs activating specific target genes involved in cell cycle arrest and apoptosis (Dohn et al., 2001; Wu et al., 2003; Liu et al., 2004).

Despite the involvement of p53 family proteins in different cellular processes, in this work a particular attention will be given to their roles on cell cycle, apoptosis and autophagy.

1.1.2.1. Cell Cycle

Cell cycle is a cellular process that is controlled by several mechanisms resulting in the transmission of the genetic information from one generation to the next. Cell cycle checkpoints (G1/S, S and G2/M) regulate the progression of cells through each stage of the cell cycle, in order to prevent the transmission of damaged genetic material to the daughter cells. These cell cycle checkpoints are regulated by a family of serine/threonine protein kinases, the cyclin-dependent kinases (CDK) (Fig. 2) [reviewed in (Vermeulen et al., 2003; Harms et al., 2004)].

The regulation of G1 phase is carried out by G1 cyclins D and E, and by CDKs 2, 4 and 6. In early G1 phase, the cyclin D forms a complex with CDKs 4 or 6, which can then target the retinoblastoma protein (Rb) for phosphorylation. Once phosphorylated, pRb binds and inhibits the E2F transcription factor. In late G1 phase, the cyclin E forms a complex with CDKs 4 or 6, which hyperphosphorylates pRB, releasing the E2F, which leads to the transcription of genes required for S-phase entry (Fig. 2). Under stress conditions, G1 arrest is induced through the inhibition of cyclin-CDK complexes by cyclin-dependent kinase inhibitors (CKIs), such as p21 and p57/Kip2. With the inhibition of these complexes, the function of E2F is compromised [reviewed in (Harms et al., 2004)].

In response to different stress signals, the p53 family proteins induce cell cycle arrest by inhibiting G1/S or G2/M transitions [reviewed in (Harms et al., 2004)]. Once activated, p53 family proteins induce the transcription of p21, a CDK inhibitor that binds to cyclins D/CDKs4/6 and E/CDKs4/6, preventing the G1/S transition. Additionally, p53 mediates the inhibition of cyclin E/CDK2 complexes by p21. In fact, to date, p21 is the only common target of the p53 family that leads to G1 arrest (el-Deiry et al., 1993; Lee and La Thangue, 1999; Dohn et al., 2001). The activity of cyclin E is also compromised through the induction of an F box protein and component of the SCF (Skp, Cullin, F-box containing complex) ubiquitin ligase complex, hCDC4b, which is a direct transcriptional target of p53 (Kimura et al., 2003). Moreover, it was described that the CDK inhibitor

p57/Kip2 is up-regulated by p63 and p73 leading to the induction of G1 arrest [reviewed in (Allocati et al., 2012)] (Fig. 2).

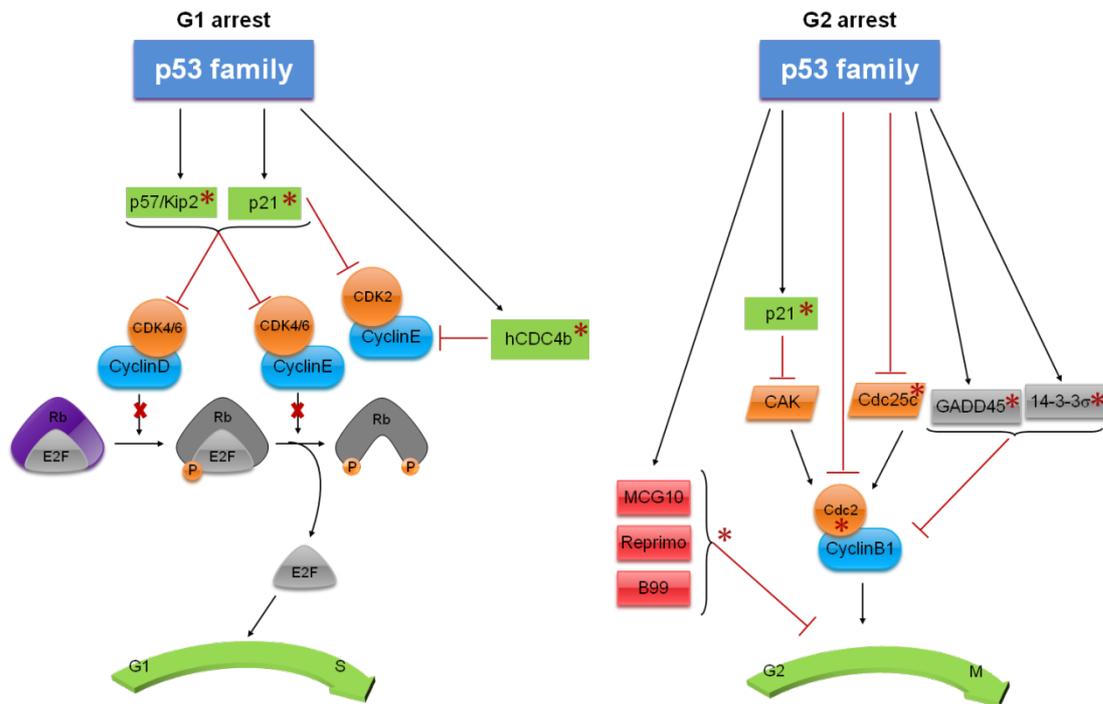


Figure 2. Cell cycle regulation by p53 family proteins. p53 family proteins induce cell cycle arrest by inhibiting G1/S or G2/M transitions. The asterisks refer to p53 family transcriptional activity [Adapted from (Harms et al., 2004)].

p53 family proteins regulate the G2/M transition by inhibiting the Cdc2, which needs to bind to cyclin B1 to be functional. Therefore, the repression of the cyclin B1/Cdc2 complex by p53 family proteins lead to a G2/M arrest (Taylor and Stark, 2001) [reviewed in (Allocati et al., 2012)]. Additionally, p53 family proteins regulate the G2/M checkpoint through modulation of the expression of multiple targets such as, Cdc25C, 14-3-3 σ , p21, and GADD45 [reviewed in (Harms et al., 2004; Allocati et al., 2012)]. Both p53 and p73 induce G2/M arrest by repressing the mitosis promoting phosphatase, Cdc25C. This phosphatase removes the phosphate groups of Cdc2 leading to the cyclin B1/Cdc2 complex activation (Taylor and Stark, 2001) [reviewed in (Allocati et al., 2012)]. Besides Cdc25C, the CDK-activating kinase (CAK) adds phosphate groups to Cdc2 on specific points that are required for the activation of the cyclin B1/Cdc2 complex [reviewed in (Harms et al., 2004)]. p21 also participates in G2 arrest through the inhibition of the cyclin

B1/Cdc2 complex by CAK (Smits et al., 2000). The growth arrest and DNA damage inducible protein (GADD45) is a p53 target gene that binds to Cdc2, preventing its binding to cyclin B1, and, consequently, the formation of the protein complex necessary for mitotic entry, which results in G2 arrest (Zhan et al., 1999). The scaffold protein 14-3-3 σ is involved in G2 arrest and is induced by both p53 and p73 and repressed by Δ Np63. In fact, this protein promotes cyclinB1/Cdc2 nuclear export. Through this mechanism, 14-3-3 σ inhibits the cyclinB1/cdc2 complex by physically separating it from its target proteins (Hermeking et al., 1997). MCG10 (a RNA-binding protein), Reprimo (a highly glycosylated protein that is thought to play a role in cyclinB1/cdc2 localization) and B99 (a protein with microtubule localization) are proteins involved in G2 arrest which expression is induced by p53 (Zhu and Chen, 2000; Ohki et al., 2000; Utrera et al., 1998) (Fig. 2).

1.1.2.2. Apoptosis

Apoptosis is a type of cell death characterized by cell shrinkage, blebbing of plasma membrane, maintenance of organelle integrity, condensation and fragmentation of DNA. There are two main apoptotic pathways: the extrinsic (or death receptor pathway) and the intrinsic (or mitochondrial pathway) [reviewed in (Indran et al., 2011; Galluzzi et al., 2012)] (Fig. 3). The extrinsic pathway consists on the activation of cell death receptors that are located at the plasma membrane (Fig. 3). This apoptotic pathway can be activated by the binding of lethal ligands, such as FAS ligand (FASL), tumour necrosis factor α (TNF α) and TNF-related apoptosis inducing ligand (TRAIL), to different death receptors, such as FAS/CD95, TNF α receptor 1 (TNFR1) and TRAIL receptor (TRAILR)1-2, respectively. These receptors have an extracellular and an intracellular cytoplasmic (also known as death domain) domain. The death domain is responsible for the death signal transmission leading to the activation of the cell death receptors. The activation of these receptors leads to their trimerization and consequent clustering of the intracellular death domain, which subsequently recruits the protein adaptor FADD (Fas-associated protein with death domain). In turn, FADD recruits the initiator, pro-caspase-8 forming the death-inducing signalling complex (DISC). The formation of the DISC complex results in the activation of pro-caspase-8 that, in turn, activates the executioner pro-caspases-3, -6 and -7, or mediates the proteolytic cleavage of BH3-interacting domain death agonist (BID), leading to the generation of a mitochondrion-permeabilizing fragment, called the truncated BID (tBID) [reviewed in (Harms et al., 2004; Indran et al., 2011; Galluzzi et al., 2012)].

On the other hand, the intrinsic pathway can be activated by different stimuli leading to alterations in the inner mitochondrial membrane permeability (Fig. 3). These alterations lead to changes at the mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and to the release of pro-apoptotic proteins [reviewed in (Indran et al., 2011)]. The pro-apoptotic proteins released are divided into two groups. The first one includes the cytochrome c (cyt c) and the direct IAP-binding protein with low isoelectric point (pI) (DIABLO; also known as second mitochondria-derived activator of caspases, SMAC), which can induce apoptosis through a caspase-dependent manner. SMAC/DIABLO inhibits the anti-apoptotic function of several members of the inhibitor of apoptosis (IAP) family, thereby activating the executioner caspases-3, -6, and -7, which will cleave nuclear and cytoskeletal structure proteins, leading to apoptosis [reviewed in (Harms et al., 2004; Elmore et al., 2007; Indran et al., 2011)]. The other group of pro-apoptotic proteins includes the apoptosis inducing factor (AIF) and the endonuclease G (EndoG), which relocate to the nucleus mediating a large-scale DNA fragmentation through a caspase-independent manner [reviewed in (Elmore et al., 2007; Indran et al., 2011, Galluzzi et al., 2012)]. This mitochondrial apoptotic pathway is controlled and regulated by Bcl-2 family members. This family consists of anti-apoptotic (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic (e.g. Bax, Bak and Bid) members. In fact, these proteins dictate whether or not cells are committed to die. Moreover, it is well established that Bcl-2 family proteins play a central role in the regulation of cyt c release from mitochondria. For instance, Bcl-2 and Bcl-xL protect the membrane integrity by interacting with mitochondrial proteins that are able to form mitochondrial pores. Therefore, this interaction leads to the inhibition of cyt c release. On the contrary, Bax can homodimerize or heterodimerize with Bak or tBib, leading to the disruption of the mitochondrial membrane integrity through the formation of mitochondrial pores that then lead to the release of cyt c [reviewed in (Indran et al., 2011)]. Once released to the cytoplasm, cyt c binds to the apoptosis protease-activating factor 1 (Apaf-1) and pro-caspase-9, forming the apoptosome. The apoptosome formation leads to pro-caspase-9 activation that, in turn, activates pro-caspases-3, -6 and -7, triggering apoptosis [reviewed in (Elmore et al., 2007; Indran et al., 2011)]. Bcl-2 family proteins include also the BH3-only proteins, such as Puma (p53 upregulated modulator of apoptosis) and Noxa. These pro-apoptotic proteins bind to the anti-apoptotic proteins blocking their effects at mitochondria [reviewed in (Elmore et al., 2007; Indran et al., 2011)].

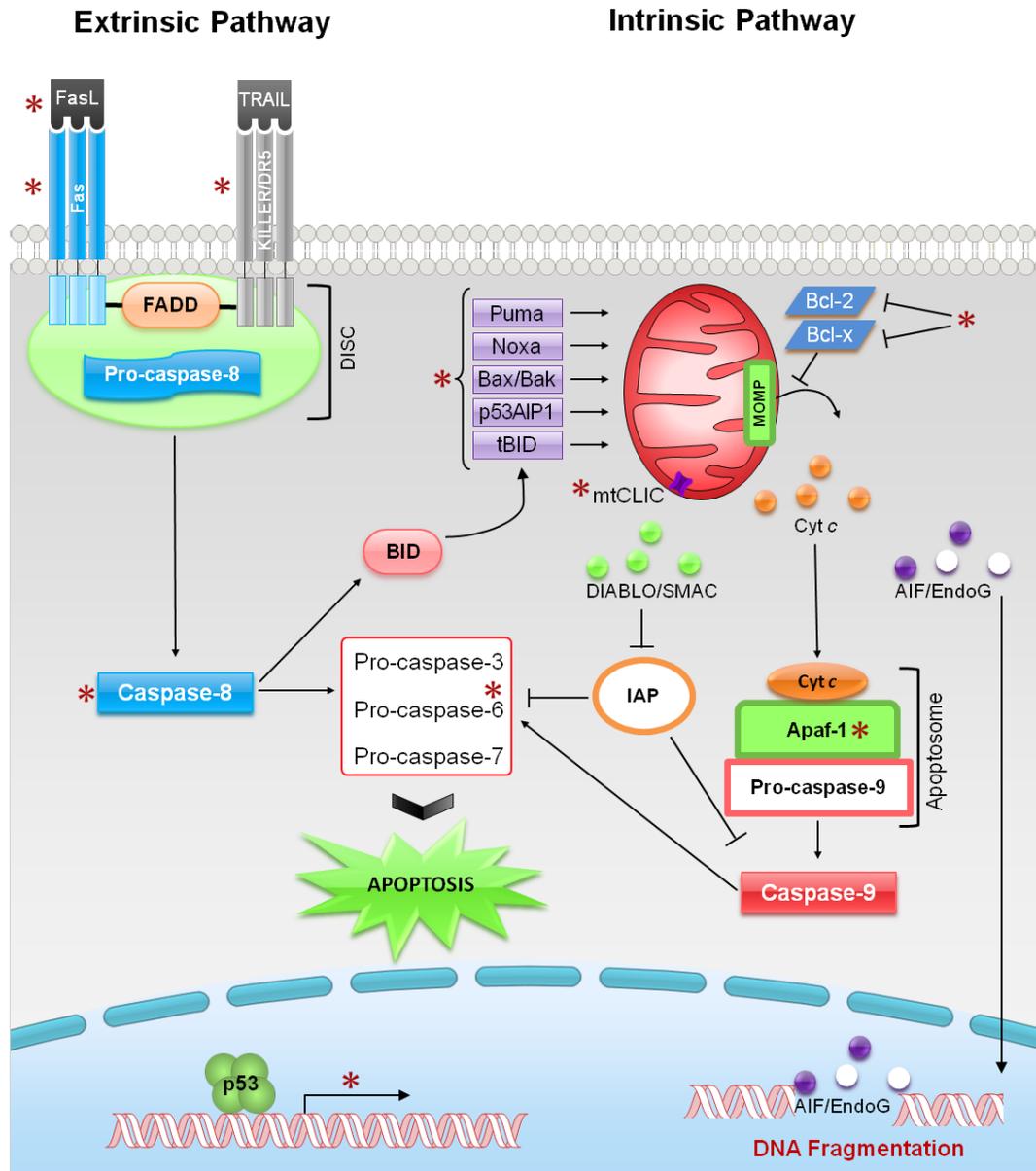


Figure 3. Intrinsic and extrinsic apoptotic pathways: regulation by p53. p53 up-regulates the expression of proteins involved in both intrinsic and extrinsic apoptotic pathways. The asterisks refer to p53 transcriptional targets [Adapted from (Indran et al., 2011)].

The p53 family proteins are able to induce apoptosis through both extrinsic and intrinsic pathways [reviewed in (Harms et al., 2004; Pietsch et al., 2008; Allocati et al., 2012)] (Fig. 3). In the extrinsic pathway, the p53 family proteins up-regulate the expression of the cell death receptor Fas/CD95 (Fukazawa et al., 1999; Gressner et al., 2005; Müller et al., 2005), of the TRAIL cell death receptors, p53 regulates KILLER/DR5 (Takimoto and El-Deiry, 2000) and p63/p73 regulate TRAIL-R1, TRAIL-R2 and TNFR1

(Gressner et al., 2005; Schuster et al., 2010), and of caspase-8 (Liedtke et al., 2003; Borrelli et al., 2009; Müller et al., 2005). In the intrinsic apoptotic pathway, p53 family proteins commonly up-regulate the pro-apoptotic proteins from of Bcl-2 family, such as Bax and Puma (Miyashita et al., 1994; Shimada et al., 1999; Nakano and Vousden, 2001; Pyati et al., 2011; Melino et al., 2004). Moreover, both p53 and p73 up-regulate Noxa (Oda et al., 2000a; Melino et al., 2002) and p53AIP1 (p53-apoptosis inducing protein 1), which localizes to the mitochondria where it interacts with Bcl-2 to facilitate the cyt c release and the consequent apoptosis induction (Oda et al., 2000b; Costanzo et al., 2002). Despite this, other apoptotic proteins exist which expression are only regulated by p53 [reviewed in (Harms et al., 2004; Elmore et al., 2007)]. For instance, p53 is able to promote apoptosis through transcriptional repression of anti-apoptotic members of the Bcl-2 family, namely Bcl-2 and Bcl-xL, which stabilize the MOMP (Sugars et al., 2001). Furthermore, p53 up-regulates Apaf-1, caspase-6 and Bid, which seems to function as a bridge between the extrinsic and intrinsic pathways (Fortin et al., 2001; MacLachlan and El-Deiry, 2002; Sax et al., 2002). After clivage by caspase-8, the tBid translocates to mitochondria participating in the intrinsic apoptotic pathway (Sax et al., 2002). p53 also transcriptionally regulates the mitochondrial chloride intracellular channel (mtCLIC/CLIC4), an organellular chloride channel protein that reduces the MOMP (Fernandez-Salas et al., 2002).

Besides its transcriptional function, accumulating data have shown that p53 can itself translocate to mitochondria, where it induces apoptosis through a transcription-independent mechanism [reviewed in (Chi, 2014)]. Briefly, in response to stress, p53 moves from the cytoplasm to mitochondria (Mihara et al., 2003), in which it forms a complex with Bcl-xL and Bcl-2 proteins. The formation of such complexes leads to the release of Bax and Bak, and to the consequent mitochondrial outer membrane permeabilization (MOMP) induction with cyt c release (Chipuk et al., 2004; Tomita et al., 2006).

1.1.2.3. Autophagy

Autophagy is a catabolic process that exerts an important role in the maintenance of cellular homeostasis. To ensure this, autophagy promotes the elimination of damaged or old organelles and degradation of long-lived proteins and protein aggregates. However, uncontrolled or excessive autophagy can lead to cell death [reviewed in (Yang and Klionsky, 2010; Napoli and Flores, 2013)]. Briefly, this catabolic process is characterized by the formation of autophagosomes, engulfing organelles and soluble factors and subsequently fusing with the lysosomes to generate the autolysosomes, where the

degradation occurs [reviewed in (Napoli and Flores, 2013)]. Autophagy was initially identified in mammals. However, the autophagy-related genes (ATGs) were first identified in yeast, and the majority of them have homologs in higher eukaryotes [reviewed in (Yang and Klionsky, 2010; Napoli and Flores, 2013)]. Most of the Atg proteins are recruited for the formation of the pre-autophagosomal structure (PAS). The formation of PAS is controlled by the mammalian target of rapamycin (mTOR) through two effector complexes: the protein kinase Atg1 (ULK1, ULK2 and ULK3 in mammals) and the lipid kinase Vps34 (hVps34 in humans). Additionally, two ubiquitylation-like conjugation systems, Atg5–Atg12–Atg16 and Atg8 (LC3 in mammals), also contribute to the formation of PAS. Autophagy induction is controlled by mTOR, which is a serine/threonine protein kinase that function as a central sensor of growth factors, nutrient signals and energy status [reviewed in (Yang and Klionsky, 2010)].

The p53 tumour suppressor protein is considered a master regulator of autophagy. Depending on its subcellular localization, p53 can activate or inhibit autophagy (Fig. 4) [reviewed in (Ryan, 2011)]. In response to stress signals, p53 is activated and, in the nucleus, it activates the adenosine monophosphate activated protein kinase (AMPK) pathway, resulting in the inhibition of the mTOR pathway and subsequent induction of autophagy (Feng et al., 2005). Besides AMPK, p53 transcriptionally regulates other pro-autophagic modulators such as death associated protein kinase 1 (DAPK-1), damage-regulated autophagy modulator 1 (DRAM1), Bax, Puma, sestrin 2 (SESN2) and tuberous sclerosis protein 2 (TSC2) [reviewed in (Sui et al., 2011; Ryan, 2011)] (Fig. 4). In addition, it was demonstrated that several autophagy-related genes, such as *Atg4*, *Atg5*, *Ulk* and *Atg7* and the two ubiquitylation-like conjugation systems Atg5–Atg12–Atg16 and Atg8 (LC3) are transcriptionally regulated by p53 (Kenzelmann Broz et al., 2013). Similarly to p53, p73 activates DRAM1. Different to the situation with p53, however, although p73 was also able to induce autophagy, this effect was DRAM-independent (Crichton et al., 2007). Additionally, the interferon stimulated exonuclease gene 20 kDa-like 1 (*ISG20L1*), which modulates genotoxic stress-induced autophagy, has been identified as a p53 family target gene (Eby et al., 2010).

Although the molecular mechanism is still not fully understood, in the cytoplasm, p53 can inhibit autophagy through repression of the AMPK pathway, resulting in the activation of mTOR (Fig. 4) (Tasdemir et al., 2008).

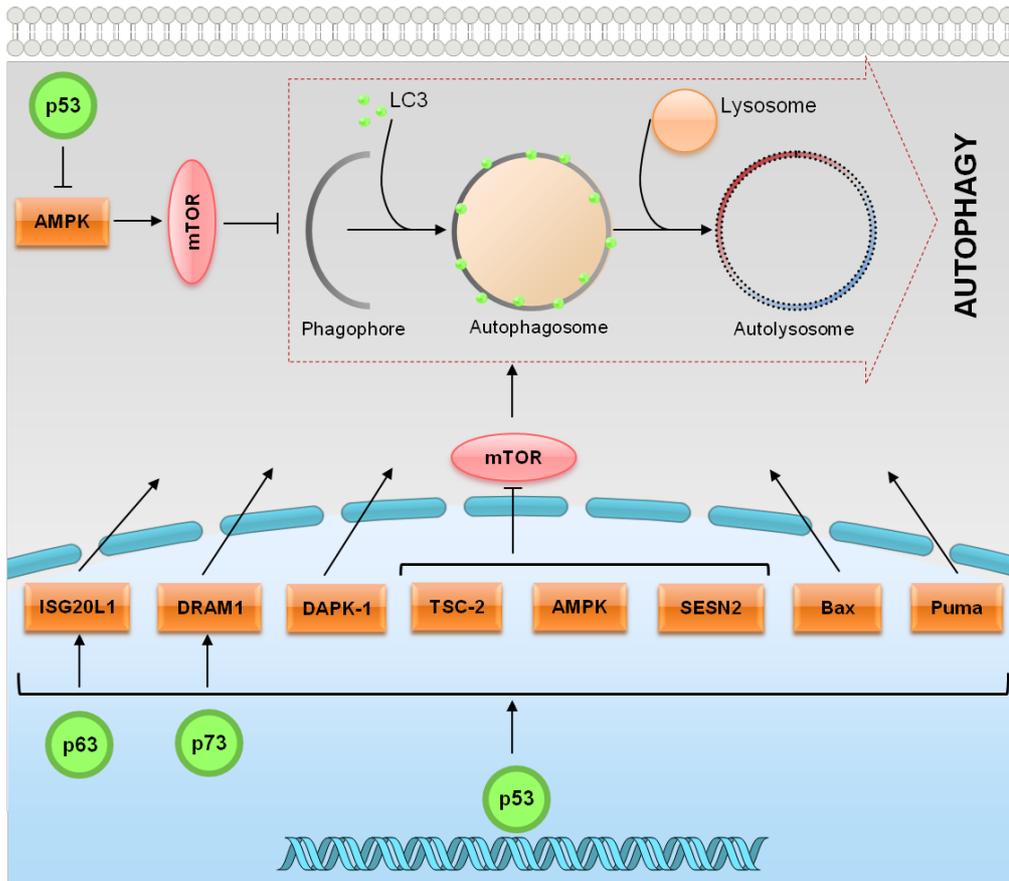


Figure 4. p53 family proteins distinctly regulate autophagy depending on its subcellular localization. In the nucleus, p53 family proteins induce the expression of proteins that activate autophagy. p53 may directly activate AMPK, TSC-2 and SESN2 and inhibit mTOR pathway, subsequently induce autophagy. p53 may also indirectly induce autophagy by binding to the promoter region of multiple genes that code for pro-autophagic modulators, such as DRAM1, DAPK-1, Bax and Puma. In the cytoplasm, p53 inhibits autophagy through repression of the AMPK pathway, resulting in the activation of mTOR [Adapted from (Ryan, 2011)].

1.1.3. Therapeutic applications

As previously referred, the activation of p53, particularly of its capacity to induce an apoptotic cell death, has been considered an attractive strategy for anticancer treatment. In half of all human tumours retaining a wt p53 form, this may be achieved through inhibition of the p53 interaction with its major negative regulators, MDM2 and MDMX (also called MDM4).

1.1.3.1. MDM2 and MDMX: Endogenous negative regulators

The MDM2 protein was identified as a product that was amplified on double minute chromosomes in transformed mouse fibroblasts (Fakharzadeh et al., 1991). Later, it was found that MDM2 was able to bind to p53 leading to the inhibition of its transcriptional activity (Momand et al., 1992; Oliner et al., 1993). Few years later, through a screening of a mouse cDNA expression library, MDMX, a homolog of MDM2, was discovered as a novel p53-interacting protein (Shvarts et al., 1996). Shvarts and colleagues (1996) demonstrated that similarly to MDM2, MDMX co-immunoprecipitates with p53 and its overexpression inhibits the p53 transcriptional activity. MDM2 and MDMX share some similarities in their structure (Fig. 5). Both proteins have a p53-binding domain at the N-terminal that is crucial for their binding to p53 and inhibition of its transcriptional activity. MDM2 and MDMX also have in common a zinc and a C-terminal RING finger domain. For MDM2, this latter domain is important for MDM2 homo-dimerization, MDM2-MDMX heterodimerization and E3 ubiquitin ligase activity. For MDMX, the RING finger domain is important for oligomerization and also gives to MDMX a minimal ubiquitin ligase activity. The major structural differences between these MDM proteins are found in the acidic domain, in the nuclear localization (NLS), and in the nuclear export signal domains (NES). For instance, MDMX has a shorter acidic domain compared to with MDM2, and lacks the NLS and NES domains (Fig. 5) [reviewed in (Waning et al., 2010)].

Regarding the *MDM2* gene, its basal and inducible expressions are controlled by P1 and P2 promoters, respectively. The P2 promoter of the *MDM2* gene is responsive to p53 induction [reviewed in (Manfredi, 2010)]. Although the obvious structural similarities between *MDM2* and *MDMX* genes, the promoter region of *MDMX* gene remains not well characterized [reviewed in (Marine and Jochemsen, 2005)]. However, recently, Phillips and colleagues (2010) reported the existence of a p53-responsive promoter (P2) in the first intron of the *MDMX* gene, thus establishing a negative regulatory feedback loop between p53 and MDMX.

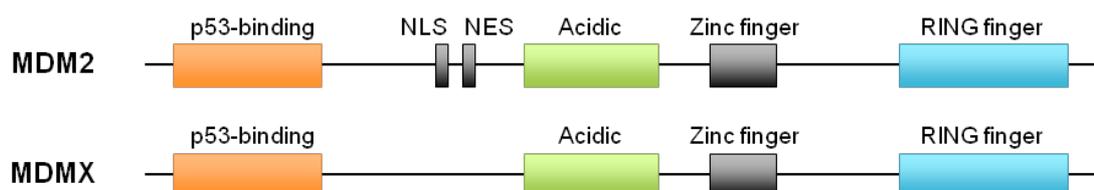


Figure 5. Schematic representation of the major MDM2 and MDMX domains. [Adapted from (Lenos and Jochemsen, 2011)].

Despite being structurally related proteins with the ability to inhibit the p53 transcriptional activity, evidence has emerged showing that these two proteins also play multifaceted and non-redundant roles in modulating p53 [reviewed in (Pei et al., 2012; Shadfan et al., 2012; Wade et al., 2013)].

Regarding the regulation of p53 by MDM2, both proteins form an autoregulatory feedback loop (Picksley and Lane, 1993; Wu et al., 1993). Once activated, p53 binds to the P2 promoter of the *MDM2* gene inducing its transcription. In turn, MDM2 binds to the N-terminus of p53, via its N-terminal hydrophobic region (Böttger et al., 1999), and negatively regulates the p53 by three different mechanisms (Fig. 6): **(1)** inhibits the p53 transcriptional activity (Momand et al., 1992); **(2)** promotes the p53 nuclear export (Freedman et al., 1999), and **(3)** leads to p53 degradation via the ubiquitin–proteasome system (Haupt et al., 1997). In fact, MDM2 leads to p53 ubiquitination either in the form of a homodimer or of a or of a MDM2/MDMX heterodimer (Uldrijan et al., 2007; Dolezelova et al., 2012). Moreover, low MDM2 expression levels lead to the monoubiquitination of p53 resulting in its nuclear export and consequent transcription inhibition. Under these circumstances, monoubiquitylated p53 can localize to the mitochondria where it is deubiquitylated by the ubiquitin-specific protease-7 (USP7). Once deubiquitylated, p53 is able to induce apoptosis through a transcription-independent mechanism. Therefore, low levels of MDM2 are able to promote p53-mediated apoptosis (Marchenko et al., 2007). On the contrary, high MDM2 expression levels lead to the polyubiquitination of p53, which results in its proteasomal degradation [reviewed in (Nag et al., 2013)]. This degradation mechanism is responsible for the maintenance of low levels of p53 in unstressed cells (Lee and Gu, 2010). MDM2 regulates the p53 ubiquitination at lysine residues present in the C-terminal of p53. Additionally, these MDM2-targeted lysine residues can also be acetylated by p300/CREB-binding protein (CBP), a protein involved in p53 activation through acetylation (Lee and Gu, 2010). Acetylation of p53 leads to its activation and promotes its transcriptional activity (Tang et al., 2008). When ubiquitinated by MDM2, p53 is not able to be acetylated by p300/CBP, which leads to its proteasome degradation (Ito et al., 2001). It was also demonstrated that the p53 ubiquitination can be abolished due to the MDM2 acetylation by p300/CBP (Wang et al., 2004).

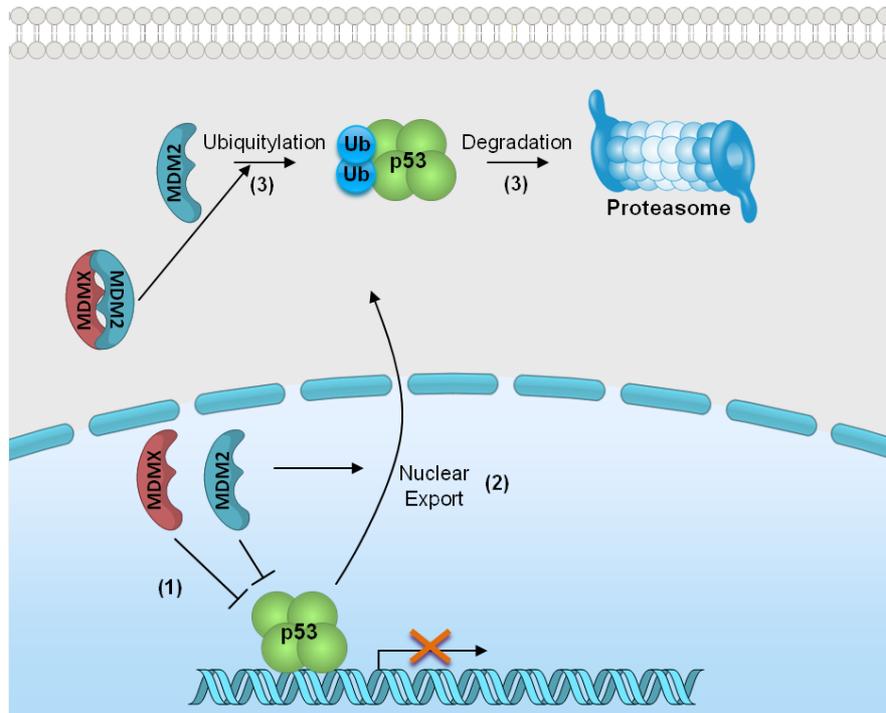


Figure 6. Regulation of p53 activity and stability by MDM2 and MDMX. MDM proteins bind to p53 and inhibit its transcriptional activity (1). MDM2 promotes the p53 nuclear export (2) and, as an E3 ubiquitin ligase, the p53 ubiquitination and proteasomal degradation (3). Ub: ubiquitin. [Adapted from (Lenos and Jochemsen, 2011)].

Similarly to MDM2, MDMX also interacts with the p53 TAD through its p53-binding domain (Böttger et al., 1999), inhibiting the p53 transcriptional activity (Shvarts et al., 1996) (Fig. 6). In fact, the binding of MDMX blocks the p53 interaction with p300/CBP what leads to the inhibition of the p53 acetylation process (Sabbatini and McCormick, 2002). As referred before, since the lysine residues that are acetylated by p300/CBP are also targeted for ubiquitination by MDM2, MDMX may indirectly stimulate MDM2-mediated p53 ubiquitination by reducing the acetylation of those lysines [reviewed in (Lenos and Jochemsen, 2011)]. Despite some ubiquitin ligase activity *in vitro*, contrary to MDM2, MDMX do not induce p53 nuclear export and degradation (Jackson and Berberich, 2000). In fact, in spite of the extensive homology of the RING finger domain of MDM2 and MDMX, MDMX has no detectable E3 ubiquitin ligase activity *in vivo* (Kawai et al., 2003; Meulmeester et al., 2003).

Some studies showed that MDMX is not only a p53 inhibitor. In fact, under stress conditions, MDMX increases the p53 stability and promotes the p53 mitochondrial apoptosis [reviewed in (Mancini et al., 2010)]. In fact, under different DNA damages the

following three events can occur: (a) MDMX is phosphorylated and is degraded by MDM2 leading to p53 activation; (b) MDMX is phosphorylated and is exported out of the nucleus, resulting in p53 activation; (c) since it was demonstrated that a fraction of MDMX was localized in mitochondria (Mancini et al., 2009), upon a lethal DNA damage, with low MDM2 levels (Shmueli and Oren 2007), MDMX promotes the mitochondrial localization of p53 phosphorylated at Ser46 and its binding to Bcl-2, with subsequent cyt c release from mitochondria and apoptosis [reviewed in (Mancini and Moretti, 2009; Mancini et al., 2010)]. These data suggest that, under stress conditions, the MDM proteins can cooperate to activate the cellular functions of p53.

Given the considerable structural homology between the TAD of p53 family proteins, it has been accepted that the MDM proteins may also bind and inhibit p63 and p73 in a similar way to what has been observed with p53. In fact, several groups have attempted to identify and characterize these possible interactions, but results have often been controversial, particularly regarding the interaction of p63 with MDM proteins [reviewed in (Collavin et al., 2010)]. Actually, by co-immunoprecipitation assays in human tumour cells, several authors reported the absence of p63 interaction with MDM2 and MDMX, as well as the absence of effect of these MDM proteins on p63 stability and transcriptional activity (Little and Jochemsen 2001; Wang et al., 2001). On the contrary, Kadakia and co-workers (2001) showed that p63 could bind to MDM2 and MDMX, leading to the downregulation of p63 transcriptional activity, but not to its degradation. In that work, it was also demonstrated that MDM2, but not MDMX, induced the p63 nuclear export, and consequently inhibited the p63-mediated apoptosis. A subsequent study reinforced the occurrence of the p63-MDM2 interaction, although the interaction led to an increase of p63 transcriptional activity (Calabró et al., 2002). More recently, the ability of p63 to interact with MDM2 and MDMX was confirmed *in vitro* using fluorescence polarization, tryptophan fluorimetry, and isothermal calorimetric titrations (Zdzalik et al., 2010). In that study, a stronger interaction of p63 with MDMX than with MDM2 was observed. Furthermore, interactions with p63 were reported to be weaker than the ones observed with p53 and p73 (Zdzalik et al., 2010). The fragility of the p63 interaction with MDM2 and MDMX interaction may explain the conflicting results obtained by distinct research groups.

Interestingly, Galli and colleagues (2010) reported that MDM2 was also able to interact with the *N*-terminally truncated isoform of p63 (Δ Np63), which lacks the TAD involved in the interaction with MDM2. Similarly to what occurs with p53, this interaction promoted the Δ Np63 nuclear export to the cytoplasm. In spite of this, MDM2 was not able to induce Δ Np63 degradation. To date, no evidence of interaction between Δ Np63 and MDMX has been reported in human tumour cells.

Contrary to p63, the interactions of p73 with MDM2 and MDMX are well characterized. It is widely accepted that these MDM proteins bind to the p73 TAD inhibiting its transcriptional activity. However, unlike to what occurs with p53, MDM2 do not promote p73 degradation (Bálint et al., 1999; Zeng et al., 1999; Wang et al., 2001). An interaction between p73 and MDM2 was also reported by Ongkeko and colleagues (1999). However, surprisingly, in that study an enhancement, rather than an inhibition, of the p73 activity was reported. Moreover, *in vitro* binding studies confirmed the ability of p73 TAD to interact with both MDM2 and MDMX (Zdzalik et al., 2010). In that study, the affinity of p73 for MDMX showed to be higher than that for MDM2. Moreover, similar binding affinities to those determined for p53 were obtained with p73 towards MDM2 and MDMX (Zdzalik et al., 2010).

Based on the exposed above, to date, it is well-accepted that MDM proteins are critical inhibitors of the p53 family activity, and the inhibition of their interactions with p53 family proteins is an undeniable therapeutic strategy against tumours [reviewed in (Lenos and Jochemsen, 2011; Li and Lozano, 2013; Zhao et al., 2013)]. Despite this, contrary to the amazing number of inhibitors of the p53-MDM2 interaction, the number and efficacy of inhibitors of the p53-MDMX interaction is very limited. Additionally, inhibitors of the p63 and p73 interaction with MDM proteins are still mostly unknown.

1.1.3.2. Regulation of p53 activity by MDM2/MDMX

In tumours retaining a wt p53 form inactivated by MDM2 and MDMX, the following strategies can be followed for an activation of p53: **(a)** modulation of MDM2 and MDMX expression; **(b)** inhibition of MDM2 E3 ubiquitin ligase activity; **(c)** inhibition of the p53-MDM2/MDMX interaction [reviewed in (Cheok et al., 2011; Essmann and Schulze-Osthoff, 2012; Wade et al., 2013; Hoe et al., 2014)].

a) Modulation of MDM2 and MDMX expression

High expression levels of MDM proteins can be found in different types of human cancers [reviewed in (Li and Lozano, 2013)]. The expression of MDM2 and MDMX can be regulated by small interfering RNA (siRNA), short hairpin (shRNA) or miRNA but more studies are required to assess the feasibility of this approach [reviewed in (Wade et al., 2013)]. Recently, two small molecules that selective inhibits MDMX expression and activated the p53 pathway inducing apoptosis were identified: the benzofuran derivative XI-006 (NSC207895) (Wang et al., 2011a) and the pseudourea derivative XI-011 (NSC146109) (Wang et al., 2011b) (Fig. 7).

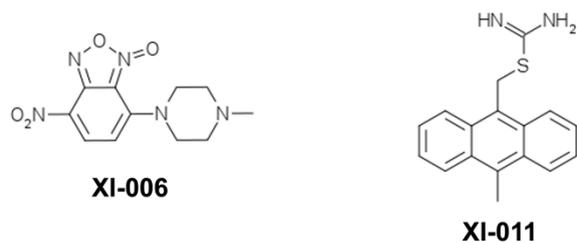


Figure 7. Chemical structures of modulators of MDMX expression.

b) Inhibition of MDM2 E3 ubiquitin ligase activity

It has been shown that the inhibition of MDM2 ubiquitin ligase activity leads to an increase of p53 expression levels in tumour cells harboring wt p53 [reviewed in (Ding et al., 2011)]. Using a high-throughput assay, Yang and colleagues (2005) identified the family pyrimido-dione-quinoline (HLI98s) as inhibitors of the MDM2 ubiquitin ligase activity (Fig. 8). Despite the capacity to increase the p53 expression levels, this family of small molecules showed low potency and solubility. In 2008, a soluble derivative of the HLI98s family, HLI373 (Fig. 8), was identified. This compound shows a high potency *in vitro*, but *in vivo* studies are still required (Kitagaki et al., 2008). Arts and colleagues (2008) demonstrated that the tryptamine derivative, JNJ-26854165 (Fig. 8), activates p53 by blocking its proteasomal degradation. However, this compound exert antitumor activity in human cancer cells with wt and mutant p53, what indicated that its mechanism of action is p53 non-specific. Despite this, due to its potent antitumor activity in cancer cells, JNJ-26854165 entered in Phase I clinical trials, and it is considered the novel first-in-clinic oral MDM2 ubiquitin ligase antagonist [reviewed in (Wade et al., 2013)]. Additionally, two natural products that inhibit MDM2 E3 ubiquitin ligase activity have been identified: Lissoclinidine B (Clement et al., 2008) and Sempervirine (Sasiela et al., 2008) (Fig. 8).

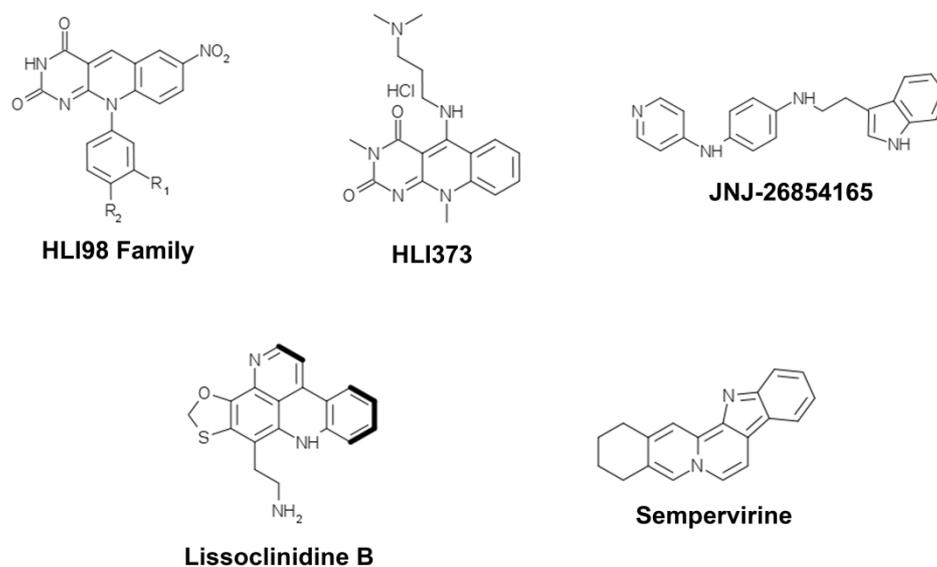


Figure 8. Chemical structures of inhibitors of E3 MDM2 ubiquitin ligase activity.

c) Inhibitors of the p53-MDMs interaction

Among the several strategies to restore the p53 activity in tumour cells, the search for small molecule inhibitors of the p53 interaction with MDMs, particularly MDM2, has been the focus of intense research.

Concerning the p53-MDM2 interaction, a huge number of small molecules from different classes have been described as inhibitors of this interaction (Fig. 9) [reviewed in (Essmann and Schulze-Osthoff, 2012; Wade et al., 2013; Hoe et al., 2014)]. The nutlin series were the first small molecules identified as inhibitors of the p53-MDM2 interaction. In 2004, Vassilev and colleagues, showed that the *cis*-imidazoline nutlin-3a fit the p53-binding pocket in MDM2, inhibiting its interaction with p53, and leading to p53 stabilization and activation. This compound triggers cell cycle arrest and apoptosis, and reduces tumour growth in a nude mice xenograft model. The derivative of nutlin-3a, RG7112 (also known as RO5045337; Fig. 9), with higher potency and improved pharmacological properties compared to nutlin-3a, has already entered in Phase I clinical trials (Ray-Coquard et al., 2012). At the same time, many other small molecules with different scaffolds, such as sulphonamides, quinolinols, terphenyls, benzodiazepinediones, spirooxindoles and isoindolinones, were identified as inhibitors of the p53-MDM2 interaction (Fig. 9) [reviewed in (Wang and Hu, 2011; Essmann and Schulze-Osthoff, 2012)], using different approaches, particularly a pharmacophore model (sulphonamide I) (Galatin and Abraham, 2004) and a fluorescence-based binding assay (quinolinole NSC66811 and

terphenyl compound 14) (Yin et al., 2005; Lu et al., 2006) (Fig. 9). Using a ThermoFluor technology, the compound 1 was identified as an inhibitor of MDM2 with a benzodiazepinedione scaffold,, which was thereafter optimized to more active compounds, TDP521252 and TDP665759 (Grasberger et al., 2005) (Fig. 9). These compounds act through a p53-dependent pathway and TDP665759 synergizes with doxorubicine treatment in a xenograft model (Koblish et al., 2006).

Using a structure-based design approach combined with molecular modelling, the spiro-oxindole scaffold was discovered as inhibitor of the p53-MDM2 interaction (Ding et al., 2005). Through sequential optimizations, the MI-219, MI-63, MI-43 and MI-319 spiro-oxindole derivatives were obtained, being MI-219 the most potent [reviewed in (Wang and Hu, 2011)] (Fig. 9). This small molecule binds to MDM2 with a high affinity and blocks the p53-MDM2 interaction, increasing the p53 levels and the expression of p53 target genes involved in cell cycle arrest and apoptosis. However, further improvement of MI-219 is required since, *in vivo*, this small molecule inhibits the xenograft tumour growth but exhibiting toxic side effects (Shangary et al., 2008). These MI compounds are in fact considered potent tryptophan-based inhibitors that bind to MDM2 with high affinity [reviewed in (Essmann and Schulze-Osthoff, 2012)].

Through an *in vitro* p53-MDM2 binding assay, several small molecules with an isoindolinone scaffold were discovered as inhibitors of the p53-MDM2 interaction (Hardcastle et al., 2005). A systematic optimization led to the identification of the active isoindolinone enantiomer 74a (Fig. 9) that activates p53, MDM2, and p21 transcription in MDM2 amplified cells and shows moderate selectivity for wt p53 cell lines in growth inhibition assays (Hardcastle et al., 2011). Thus, more studies are underway to study the selectivity and drug-like properties for this class of inhibitors.

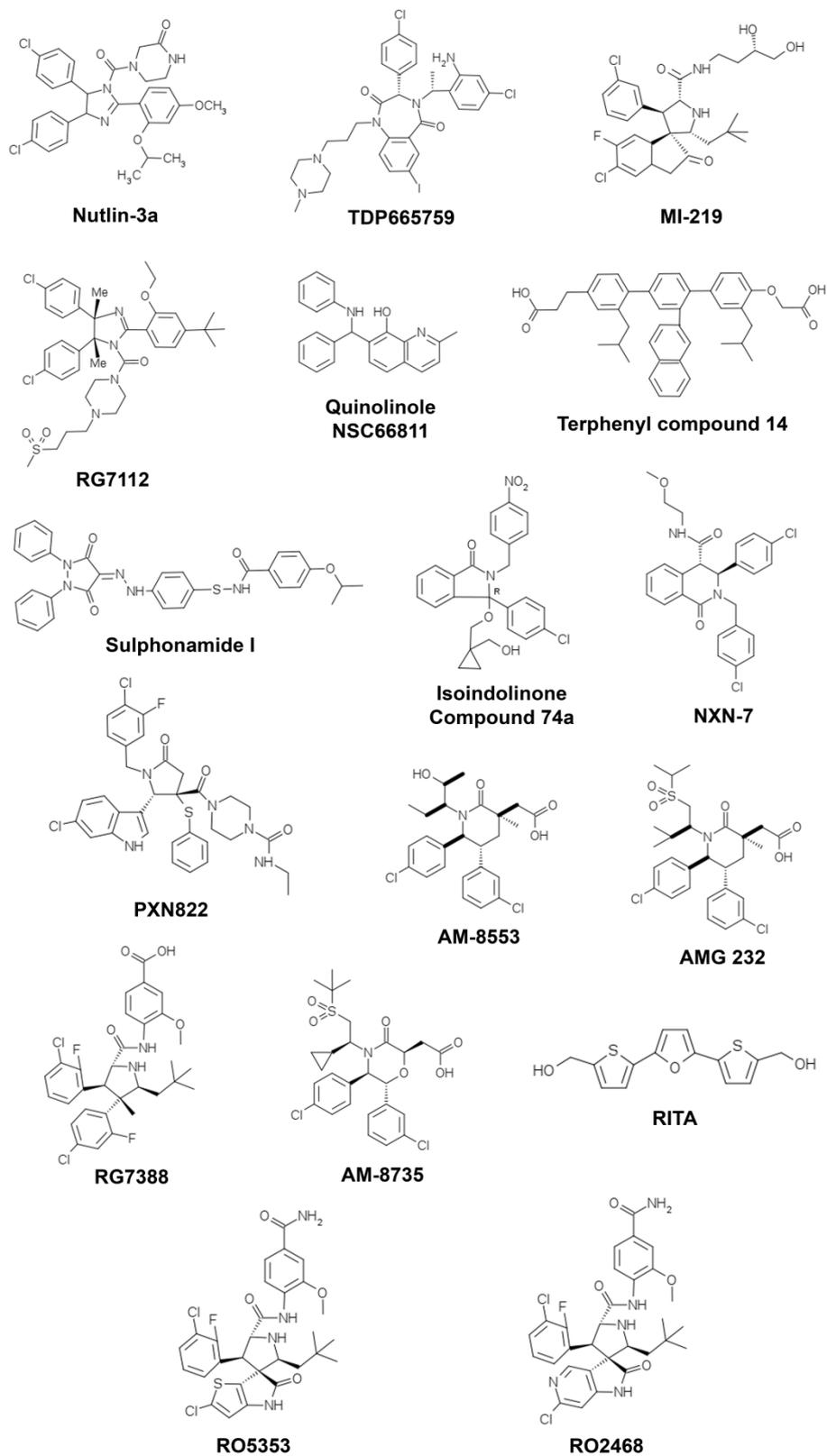


Figure 9. Chemical structures of inhibitors of the p53-MDM2 interaction.

Compounds with a pyrrolidine-2-one scaffold, such as PXN727 and PXN822, were also identified as inhibitors of the p53-MDM2 interaction. In fact, it was demonstrated that PXN822 (Fig. 9) induces p21 and Puma expression *in vitro* and inhibits tumour growth in a mouse xenograft model (Burdack et al., 2010).

From a homogeneous time-resolved fluorescence (HTRF)-based high-throughput screening (HTS) of a chemical library of chromenotriazolopyrimidine inhibitors of the p53-MDM2 interaction were identified (Allen et al., 2009; Beck et al., 2011). Based on this previous research, a structure-based rational design was developed based on the binding mode of nutlin-3a, MI-63 and chromenotriazolopyrimidine to MDM2, what led to the identification of the p53-MDM2 interaction inhibitor piperidinone AM-8553 (Bernard et al., 2012) (Fig. 9). This compound was further optimized and resulted in the piperidinone AMG 232 (Fig. 9), a highly potent MDM2 inhibitor with pharmacokinetic properties and *in vivo* antitumour activity in a xenograft model (Sun et al., 2014). Changing the piperidinone core to a morpholinone core of AMG 232, compounds with higher potency and metabolic stability, namely the AM-8735, were obtained (Gonzalez et al., 2014) (Fig. 9). This inhibitor AM-8735 presents a good pharmacokinetic profile and shows a potent *in vivo* antitumour activity (Gonzalez et al., 2014).

Recently, Ding and colleagues (2013) identified the pyrrolidine scaffold as an inhibitor of the p53-MDM2 interaction. This novel core emerged based on the structure design approach performed for the spiro-oxindoles class (Ding et al., 2005) and on the X-ray crystal structures performed for MI-219 and MI-63 with MDM2 (Popowicz et al., 2010). From a library of small molecules with a pyrrolidine core, RG7388 (Fig. 9) emerged as a selective MDM2 antagonist that inhibits the binding of MDM2 to p53, inducing cell cycle arrest and apoptosis in human tumour cells (Ding et al., 2013). Additionally, RG7388 leads to tumour growth inhibition or regression *in vivo*. The mechanism of action of this compound is similar to that of RG7112, although with a higher potency and selectivity. RG7388 is undergoing clinical investigation in solid and hematological tumours (Ding et al., 2013). Followed several optimizations of RG7388, the small molecules RO5353 and RO2468 (Fig. 9) were identified as more potent, selective and orally active p53-MDM2 inhibitors. Both compounds demonstrated a high efficacy against human osteosarcoma xenografts in mice (Zhang et al., 2013).

Most of the small molecules identified bind to MDM2 and disrupt the p53-MDM2 interaction. However, the furan derivative RITA (reactivation of p53 and induction of tumour cell apoptosis) (Fig. 9) is able to block such interaction by binding to p53 and inducing conformational changes in the protein that prevents its interaction with MDM2. In spite of this, an *in vitro* study indicated that RITA did not interfere with the p53-MDM2 interaction (Krajewski *et al.*, 2005), what gave rise to some controversy around its

mechanism of action [reviewed in (Essmann and Schulze-Osthoﬀ, 2011; Wade et al., 2013)].

During the last years, most of the pharmacological eﬀorts have been focused on MDM2. On the contrary, to date, a very low number of inhibitors of the p53-MDMX interaction have been identified. Moreover, it has been shown that inhibitors of the p53-MDM2 interaction, such as nutlin-3a are largely ineﬀective against tumours overexpressing MDMX, such as human retinoblastomas and melanomas (Wade and Wahl, 2009). Actually, diﬀerences in their p53 binding pockets justify that small molecules optimized for MDM2 have low aﬃnity for MDMX (Popowicz et al., 2007). Through X-ray crystallographic studies, Popowicz and colleagues (2010) reported the first small molecule-MDMX co-crystal structure. The small molecule WK298 (or Novartis 101) (Fig. 10) binds to MDMX in the p53 binding pocket and is therefore considered an inhibitor of the p53-MDMX interaction. However, the cell activity remains unavailable for this compound. At the same time, using an *in vitro* fluorescence polarization binding assay, Reed and colleagues (2010) identified the small molecule inhibitor of the p53-MDMX interaction called SJ-172550 (Fig. 10). This compound binds to MDMX in the p53 binding pocket inducing cytotoxic eﬀects in human tumour cells in a p53-dependent manner.

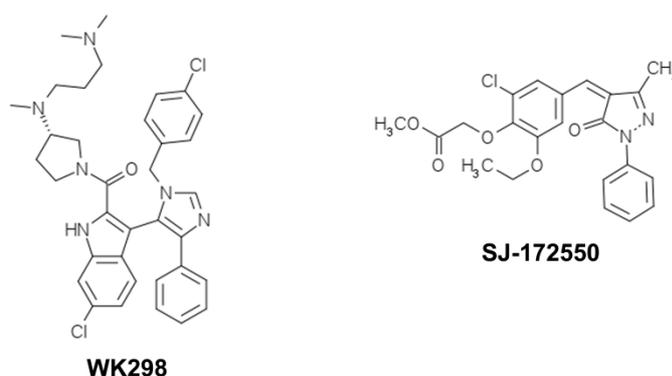


Figure 10. Chemical structures of the inhibitors of the p53-MDMX interaction.

The development of dual inhibitors of the p53 interaction with both MDM2 and MDMX has emerged as a promising therapeutic strategy for a full p53 reactivation in tumours retaining a wt p53 [reviewed in (Pei et al., 2012; Shadfan et al., 2012; Wade and Wahl et al., 2013)]. To date, only two dual inhibitors of MDM2 and MDMX were reported. Using a fluorescence polarization-based assay, the pyrrolopyrimidine-based compound 3a (Fig. 11) was identified as a dual inhibitor of p53-MDM2/MDMX interactions, which increases p53 and p21 expression levels and triggers apoptosis through a p53-dependent

pathway (Lee et al., 2011). More recently, Graves and colleagues (2012) identified the indolyl hydantoin RO-2443 that binds to the p53-binding pockets of MDM2 and MDMX by inducing the formation of dimeric protein complexes. Despite its potent MDM2 and MDMX inhibitory activity *in vitro*, RO-2443 has poor solubility. To circumvent this problem, this small molecule was optimized given rise to RO-5963 (Fig. 11) with only a slight increase of potency, but high solubility (Graves et al., 2012). In tumour cells, RO-5963 leads to a p53-dependent cell cycle arrest and apoptosis. Moreover, despite the similar effects *in vitro*, RO-5963 was more effective inhibitor of the p53-MDMX interaction than of the p53-MDM2 interaction.

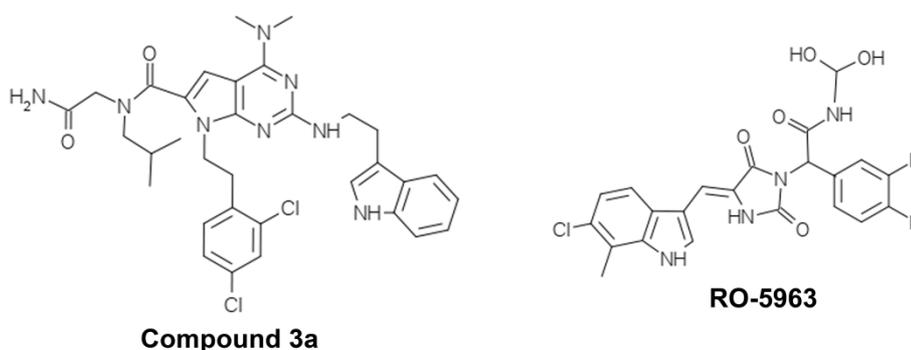


Figure 11. Chemical structures of the dual inhibitors of the p53-MDM2/MDMX interaction.

1.1.3.3. Regulation of p63 and p73 activity by MDM2/MDMX

Given that both p63 and p73 are rarely mutated in human cancers, an appropriate regulation of p63 and p73 pathways represents an important anticancer therapeutic strategy, particularly in tumours with null-p53 or with a mutant p53 form [reviewed in (Wang and Sun, 2010; Bisso et al., 2011; Wei et al., 2012; Candi et al., 2014)].

Emerging evidence showed that nutlin-3a inhibited the growth and induced apoptosis in p53-null human tumour cells (Lau et al., 2008; Peirce and Findley, 2009). This p53-independent effect of nutlin-3a was attributed to possible p73 activation, through inhibition of its interaction with MDM2. In fact, similarly to what was observed with the p53-MDM2 interaction, it was demonstrated that nutlin-3a also disrupted the p73-MDM2 interaction, enhancing the p73 activity (Lau et al., 2008). In spite of this, besides nutlin-3a, no more inhibitors of the p73 interaction with MDM proteins have been described so far.

Concerning p63, to date, no small molecule inhibitors of its interaction with MDM proteins have been described [reviewed in (Wang and Sun, 2010)].

1.2. YEAST AS A CELL MODEL

1.2.1. Yeast as a cell model to study human proteins

The high degree of conservation of cellular processes and molecular pathways with human cells has made the yeast *Saccharomyces cerevisiae* a powerful cell model to study human proteins and the molecular mechanisms underlying the pathobiology of several human diseases. In fact, the yeast cell system presents many advantages over other cell models, particularly: *a*) easy and low-cost manipulation and short generation time; *b*) well-defined genetic system and high amenability to genetic modifications; *c*) possibility for an independent analysis of a wide range of human proteins (or simplified networks of human proteins) without orthologues in this microorganism. Despite this, it must be noted that as a unicellular organism several aspects of human disorders that rely on multicellularity and cell-cell interactions are difficult to evaluate. Moreover, as a simplified cell system, some genes involved in the pathobiology of the human disease may not be present in the yeast genome (Botstein and Fink, 1988; Smith and Snyder, 2006; Hartwell, 2002; Barberis et al., 2005). Still, although the obvious limitations of using yeast to study human disorders, when used in complementarity with higher eukaryotic cell systems, yeast may greatly contribute to the uncovering of the pathobiology of human disorders, as well as to the discovery of new therapeutic opportunities.

If the gene implicated in the human disease is conserved in yeast, its function can be directly studied in this organism. However, if the gene implicated in the human disease has no orthologues in yeast, its heterologous expression in this microorganism will allow the study of the human protein in a simplified eukaryotic environment, without the interference of other proteins with similar functions, endogenous regulators and redundant processes (Loewith et al., 2002; Coutinho et al., 2009b). This so-called humanized yeast model has been widely used by our group for biological and pharmacological studies of human proteins with a key role in cancer progression.

1.2.2. Yeast as a valuable screening tool in anticancer drug research

Besides its crucial contribution to the clarification of several complex cellular processes, in recent years, due to several advantages over other screening tools (Table 1), yeast has emerged as a valuable cell system in genetic and chemical large-scale screenings [reviewed in (Menacho-Marquez and Murguia, 2007; Barberis et al., 2005; Simon and Bedalov, 2004)]. In fact, the yeast screening assays have been greatly useful in the first-line screening of potential active compounds to be tested in more complex cell models. The use of these assays in the early phase of the screening may greatly reduce the costs and may expedite the discovery of new therapeutic agents.

Table 1. Advantages and drawbacks of yeast as a screening tool over cell-free and human cell-based assays.

Yeast cell-based assays	
Advantages	
Over cell-free assays	Over human cell-based assays
Purification of the target protein is not required	A clean read-out in a null background environment for the expression of the human protein
Target proteins are examined in a cellular context (the most natural physiological state)	Less expensive to culture
Selection against compounds that are generally cytotoxic, instable in cell culture or that cannot permeate plasma membrane.	Faster growth (lower duplication time) in defined media
	Easier to propagate in HTS automated systems
	Versatile genetic malleability
	Multitude of genetic tools and cellular selection systems readily converted to HTS formats: advanced plasmid systems, homologous recombination techniques and selection of easily-scored markers
Drawbacks	
Are not the most physiologically relevant model system =>> tests in mammalian cells are ultimately essential to validate the pharmacological relevance of the targets identified	
Remarks	
Cell wall does not limit compound permeability, which is similar to mammalian cells, and the yeast pleiotropic drug resistance exporter proteins are structurally similar to the mammalian multiple drug resistance efflux pumps	

For long, yeast has been employed in drug discovery through the identification of compounds that cause a desirable physiological change, rather than modulate a specific

protein. However, a limitation of this drug discovery strategy, which does not begin with a search against a specific target, is that the precise mechanism of action cannot be determined without first identifying the molecular target, which is frequently a costly and complex task. The possibility of reproducing the function of disease-associated human proteins in the cellular environment of this organism led to the development of target-directed assays, in opposition to the non-target-based strategy [reviewed in (Menacho-Marquez and Murguia, 2007; Barberis et al., 2005; Simon and Bedalov, 2004)].

The most common procedure in yeast target-directed assays is the gene overexpression. This technique is quite popular in yeast and widely used by our group, consisting in the use of a high-copy library, in which a cDNA is expressed under the control of a regulatable promoter. In the last years, overexpression assays have been widely applied to human disease-associated proteins that induce a screenable phenotype, like growth arrest. In fact, most of the human proteins involved in cancer are toxic when overexpressed in yeast by interfering with growth-regulatory pathways (e.g. p53 and caspases). Based on this, the restoration of yeast cell growth is the basis of several chemical and genetic screenings for inhibitors of human target proteins. Likewise, the increase of the growth arrest induced by the foreign protein is the basis of several screenings for activators of human target proteins. These assays can be easily adapted to HTS based on simple measurements of the yeast cell growth by optical density. In fact, several works have already demonstrated the effectiveness of engineering yeast cells in the development of fast, selective and cost-effective HTS assays (Gelperin et al., 2005; Sopko et al., 2006; Hu et al., 2007). Despite this, other yeast target-based screening approaches have been proposed as complementary or even as an alternative to the cell growth systems. One example are those assays based on the use of reporter systems (e.g. *LacZ* and Luciferase).

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

Concerning the PKC family proteins, several growth-inhibitory assays using yeast cells expressing individual isoforms of this family were developed. With these assays, the potency and isoform-selectivity of well-known PKC activators and inhibitors were characterized. Additionally, new PKC modulators were discovered [reviewed in (Silva et al. 2012)], namely the diterpene compound 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U), a potent and selective activator of novel PKC δ and ϵ with antiproliferative effects against several human tumour cell lines (Coutinho et al., 2009a)

[Portuguese Patent (Saraiva et al., 2009)]. In this work, contrary to phorbol 12-myristate 13-acetate, which activates classical and novel PKCs inducing their translocation to the plasma membrane and a G2/M cell cycle arrest, coleon U only activated the novel PKC δ and ϵ inducing their translocation to the nucleus and a metacaspase- and mitochondria-dependent apoptosis. Interestingly, these results confirmed that, as in mammalian cells, also in yeast distinct stimuli promoted the translocation of PKCs to different cellular compartments and subsequently the induction of distinct cellular responses.

A similar growth-inhibitory assay to that of PKC isoforms was also developed for human caspase-3 and -7. In fact, using yeast cells individually expressing these caspases, new small molecule inhibitors of caspase-3 (Glória et al., 2011) and activators of caspase-7 (Pereira et al., 2014) were identified from the screening of large libraries of aspartic vinyl sulfones and flavonoids, respectively. The identified compounds may pave the way for a new class of small molecule modulators of caspase-3 and -7 with promising anticancer properties.

1.2.3. Yeast as a cell model to study p53 family proteins

The high complexity of the p53 family pathway is in part due to the coexistence of several members in a same mammalian cell. The versatility of the yeast cell model for studying target human proteins has justified the attractiveness of this cell system for an individual analysis of p53 family proteins.

Indeed, despite the complexity of p53 regulation in higher eukaryotes, at the beginning of the 1990s, it was shown that human wt p53 was also a sequence-dependent transcription factor in yeast (Fields and Jang, 1990; Shärer and Iggo, 1992). Subsequently, additional evidence was provided corroborating the remarkable similarities between the p53 transcriptional activity in yeast and mammalian cells (Yousef et al., 2008). With this interesting discovery, yeast became a powerful tool for uncovering major aspects of the function of p53.

Ishioka and colleagues (1993) developed the technique of functional analysis of separated alleles in yeast (FASAY) with the aim of understanding the status of p53 in tumour cells. Several modified versions of this FASAY assay were subsequently used to identify tumour-derived p53 gene mutations and to understand how these mutations could interfere with the p53 function in human tumour cells (Flaman et al., 1995; Balmelli-Gallacchi et al., 1999). Moreover other modified versions of FASAY assay were used to compare the transcriptional activity of p53 and p63 on *CDKN1A*, *BAX* and *MDM2* response elements (REs) (Shimada et al., 1999) and to analyse the ability of the p53

mutants to inhibit the p53 transcriptional activity (Monti et al., 2003). Another type of yeast-based p53 transactivation assay was recently developed (Andreotti et al., 2011). This highly defined dual-luciferase p53 functional assay was developed in *S. cerevisiae* based on a previous system, and was designed to address functions of p53 mutants and target response elements by varying the p53 levels (Resnick and Inga, 2003; Jordan et al., 2008). This genetically well-defined and cost-effective assay can be used in parallel to mammalian cell-based assays for investigating the effectiveness of small molecules either on wt and mutant p53 transactivation potential or on the functional interaction between wt p53 and cofactors such as MDM2 and 53BP1 (Fig. 12). The effectiveness of this assay was validated with two known inhibitors of the p53-MDM2 interaction, RITA (Issaeva et al., 2004) and Nutlin-3a (Vassilev et al., 2004). Similarly to that observed in mammalian cells, both RITA and Nutlin-3a could relieve the MDM2-dependent inhibition of wt p53 transactivation function. It was also shown that MDM2 can interact with endogenous yeast pathways to ubiquitylate and sumoylate p53. Ubiquitylation led to p53 degradation, whereas sumoylation was essential for the localization of p53–MDM2 complexes in yeast nuclear bodies (Di Ventura et al., 2008).

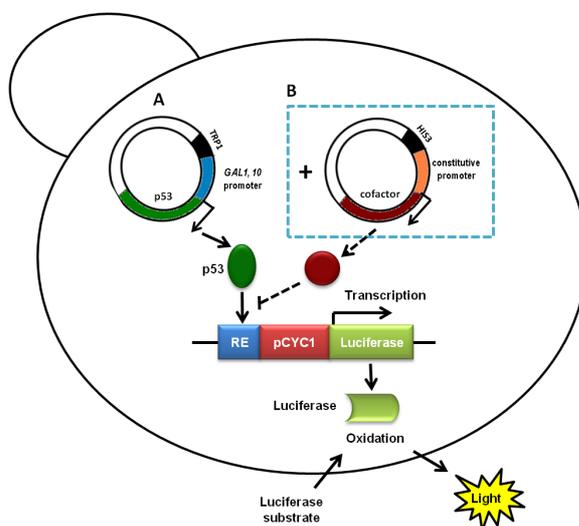


Figure 12. Yeast-based p53 dual-luciferase transactivation assay using cells. These assays can use yeast cells (A) expressing human wt/mutant p53 or (B) co-expressing human wt p53 and a cofactor (e.g. MDM2 or 53BP1). The assay exploits the variable expression levels of p53 protein and utilizes the *Firefly* and *Renilla* luminescent reporters integrated as single copies at different chromosomal loci in haploid strains or at the same chromosomal location in diploid strains (heteroalleles). While a common minimal promoter controls low-level constitutive expression of both reporters, p53-dependent expression of the *Firefly* reporter is attained through a specific p53 RE placed upstream of the minimal promoter (pCYC1). The p53 transcriptional activity is evaluated

by quantification of luciferase activity used as reporter gene, which is directly proportional to the light units measured in a plate reader.

Moreover, yeast-based transactivation assays based on the use of the reporter Luciferase were developed for p63 and p73 (Ethayathulla et al., 2012; Ciribilli et al., 2013; Raimondi et al., 2013). Using different REs, Ciribilli and colleagues (2013) demonstrated that p53, p63 and p73 have overlapping transactivation profiles in yeast. More recently, using the same yeast transactivation assay, Monti and colleagues (2014) demonstrated that both TAp63 α and Δ Np63 α are transcriptionally active in yeast exhibiting intrinsic differences in transactivation specificities.

Yeast was also used to study interactions between p53 family proteins and other proteins. The yeast two-hybrid (Y2H) system is the best known genetic assay in yeast. It was developed more than 20 years ago as a tool to identify and characterize protein-protein interactions (Fields and Song, 1989). This assay was developed in *S. cerevisiae* taking advantage of the properties of the transcriptional factor *GAL4*, which is composed by two domains, a DNA-binding domain (DBD) and a transactivation domain (AD) that can be separated and individually fused to the interest proteins. Upon co-expression, protein-protein interaction is detected because reconstitution of the transcription factor leads to the expression of a reporter gene [reviewed in (Lentze and Auerbach, 2008)]. This assay was important to determine the amino acids of MDM2 that are critical for binding to p53 (Freedman et al., 1997). This method was also important to uncover not only the interaction between the anti-apoptotic protein translationally controlled tumour protein (TCTP) and p53, but also the critical binding sites between these two proteins (Rho et al., 2011). Binding is important for TCTP to destabilize p53, preventing it to induce apoptosis which may lead to malignant transformation (Rho et al., 2011). Y2H assay were also used to study the interactions between Δ Np63 and wt/mutant p53 (Ratovitski et al., 2000) and between TAp63 isoforms with other p53 family members (Kojima et al., 2001). Additionally, Kojima and colleagues (2001) used the Y2H method to analyse the affinity between p63/p73 and MDM2/MDMX.

Yeast has also been used to understand the role of p53 tumour suppressor protein on cell growth and cell cycle. In fact, the induction of growth arrest by human wt p53 expressed either in *S. cerevisiae* (Nigro et al., 1992) or *S. pombe* (Bureik et al., 1997) has been highly explored. In *S. cerevisiae*, it was reported that wt p53 caused a mild growth inhibition (Nigro et al., 1992; Mokdad-Gargouri et al., 2001) which was markedly increased by the co-expression of human cell cycle regulated protein kinase (Nigro et al., 1992).

These results were confirmed by a latter study, in which it was observed that the wt p53-induced growth inhibition in *S. cerevisiae* was associated with S-phase cell cycle arrest (Coutinho et al., 2009b). Interestingly, with the exploitation of the p53 phenotype in *S. pombe*, it was shown that the p53-induced cell cycle arrest was abrogated by the cell cycle regulator protein phosphatase *cdc25* (Bureik et al., 1997). In spite of this, Amor and colleagues (2008) reported that the expression of wt p53 in *S. cerevisiae* resulted in apoptosis. It was also revealed that p53 interfered with the expression of the gene encoding the anti-apoptotic and anti-oxidative protein, thioredoxin. Because this protein has a crucial function in the protection of yeast cells against reactive oxygen species (ROS), these results suggested that p53 may induce apoptosis in part by downregulation of anti-apoptotic proteins.

The validation of the yeast cell system to search for p53 inhibitors was demonstrated by using the selective p53 transcriptional inhibitor pifithrin (PFT)- α that completely abrogated the p53-induced growth arrest (Coutinho et al., 2011). The regulation of p53 by PKC isoforms was also studied using the yeast cell model. Using yeast cells co-expressing PCK α , δ , ϵ or ζ and wt p53, the role of individual PKC isoforms in the regulation of p53 activity was analysed in unstressed (Coutinho et al., 2009b) and under stress conditions (Coutinho et al., 2011). These studies revealed a differential regulation of p53 by PKC isoforms. Moreover, the study performed by Coutinho and colleagues (2011) was the first evidence for the conservation in yeast of a transcription-independent p53-mediated apoptosis. In fact, in that study, it was shown that the p53 translocation to mitochondria was regulated in yeast by natural (PKC δ and ϵ) and chemical (PFT- μ) regulators. While, PKC δ and ϵ stimulated the p53 translocation to mitochondria, PFT- μ inhibited that translocation. With these results new insights about the regulation of p53-independent transcriptional activity by PKC isoforms was suggested for the first time.

Together, the evidences provided above highlight the yeast cell model as a powerful tool for biological and pharmacological studies of p53 family proteins.

1.3. SCOPE OF THIS THESIS

As addressed in previous section, p53 family proteins are major therapeutic targets in cancer. As such, a better understanding of the biology of these proteins has been the focus of an intense research since it holds the key to future therapeutic benefits. However, despite the great advances in the field of p53 family proteins, several aspects of their biology, particularly of p63 and p73, are still unclear and controversial, in part due to the extraordinary complexity of the p53 family pathway in human cells. Likewise, the pharmacological profile of p63 and p73 remains mostly unknown. Even for p53, to date, most of the pharmacological efforts have been focused on MDM2. In fact, inhibitors of MDMX, as well as dual inhibitors of MDM2/MDMX are largely missing. Additionally, despite the huge number of inhibitors of the p53-MDM2 interaction reported in the last years, only few have reached clinical trials. Therefore, further inhibitors of the p53-MDM2 with improved pharmacological properties are still required.

Based on the exposed above, with this thesis, it was intended to:

- i) Study the biology and pharmacology of p53 family proteins;
- ii) Develop target-directed screening approaches to search for modulators of individual p53 family proteins;
- iii) Identify new small-molecule modulators of p53 family proteins.

With these goals, the yeast *Saccharomyces cerevisiae* was selected as a cell model system. In this thesis, the study of the regulation of p53 by MDM2 in yeast led to the development of a new screening assay for the search of inhibitors of the p53-MDM2 interaction (Chapter 3.1). With the developed yeast p53-MDM2 interaction assay, a new small molecule inhibitor of the p53-MDM2 interaction with a xanthone scaffold (pyranoxanthone 1; LEM1) was discovered (Chapter 3.1), and new insights concerning the molecular basis of activity, as potential inhibitors of the p53-MDM2 interaction, of α -mangostin and gambogic acid were provided (Chapter 3.2). The regulation of p53 by MDMX in yeast was also assessed in this thesis, which led to the development of a new screening approach to search for inhibitors of the p53-MDMX interaction (Chapter 3.3). With the identification of *ACT1* as an endogenous p53 target gene in yeast, a new simplified yeast p53 transactivation assay, not based on artificial reporter constructs, was developed for the analysis of the p53 transcriptional activity (Chapter 3.3). Besides p53, the effect of p63 (full length and truncated forms) and p73 on cell proliferation and death, and their regulation by MDM2 and MDMX was also analysed in yeast. This study led to the development of new screening assays for the search of modulators of p63 and p73 activity, particularly of inhibitors of the p63 and p73 interaction with MDM2/MDMX (Chapter 3.4). With the developed yeast p73-MDM2 interaction assay, a putative small

molecule inhibitor of the p73-MDM2 interaction with a xanthone scaffold (LEM2) was identified (Chapter 3.5).

The chapter 4 of this thesis consists of a general discussion and conclusions focusing on the major contributions of this work to advance our knowledge about the biology and pharmacology of p53 family proteins, as well as to the development of new screening approaches that may expedite the discovery of new regulators of p53 family proteins with potential therapeutic applications.

Chapter 2

Materials and Methods

2.1. COMPOUNDS

Nutlin-3a was obtained from Alexis Biochemicals (Grupo Taper, Sintra, Portugal). Doxorubicin, SJ-172550, rapamycin and pifithrin- α (PFT- α) were obtained from Sigma-Aldrich (Sintra, Portugal). The xanthone derivatives were investigated: 3,4-dihydro-12-hydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3,2-*b*]xanthen-6-one (**1**), 3,4-bis-(3-methylbut-2-enyloxy)-9*H*-xanthen-9-one (**2**), 3-(3-methylbut-2-enyloxy)-4-hydroxy-9*H*-xanthen-9-one (**3**), (\pm)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-11*H*-1,4-dioxino[2,3-*b*]xanthen-11-one (**4**), 1-(6-bromohexyloxy)-9*H*-xanthen-9-one (**5**), 3,4-dihydroxy-9*H*-xanthen-9-one (**6**), 3,6-dihydroxy-9*H*-xanthen-9-one (**7**), 1-carbaldehyde-4-hydroxy-3-methoxy-9*H*-xanthen-9-one (**8**), 2,2',4,4'-tetracetylbenzophenone (**9**), 2-(((*S*)-oxiran-2-yl)methoxy)-1-hydroxy-9*H*-xanthen-9-one (**10**), 1,3-dihydroxy-2-methyl-9*H*-xanthen-9-one (**11**), 2,3-dihydro-10-hydroxy-3,3-dimethyl-11-(2-methylbut-3-en-2-yl)pyrano[2,3-*c*]xanthen-7(1*H*)-one (**12**), 4-hydroxy-1((isobutylamino)methyl)-3-methoxy-9*H*-xanthen-9-one (**13**), 2,3-dihydro-6-hydroxy-3,3,5-trimethylpyrano[2,3-*c*]xanthen-7(1*H*)-one (**14**) and 1-carbaldehyde-3,4-dimethoxy-9*H*-xanthen-9-one (**LEM2**) were obtained and characterized according to described procedures (Pedro et al., 2002; Sousa et al., 2002; Castanheiro et al., 2007; Sousa et al., 2009; Costa et al., 2010; Palmeira et al., 2010; Palmeira et al., 2011; Paiva et al., 2012). Details concerning the extraction of α -mangostin (**15**) have been previously reported (Marquez-Valadez et al., 2009). The purity of compound (**15**) was measured by HPLC and was of 98%. Gambogic acid (**16**) was from Sigma-Aldrich (Sintra, Portugal). All tested compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Sintra, Portugal).

2.2. YEAST CELLS

2.2.1. Plasmids

The plasmids used in the yeast assays are presented in the Table 2.

2.2.2. Yeast strains

The *Sacharomyces cerevisiae* strains used in the yeast assays are presented in Table 3.

Table 2. Yeast expression vectors used in the yeast assays.

Plasmid	Protein	Supplier	Promoter
pGADT7-(<i>LEU2</i>)	MDM2	Dr. X.M. Zhang, National Center of Biomedical Analysis, China	Constitutive (<i>ADH1</i>)
	MDMX	Dr. M. Scheffner, University of Konstanz, Germany	
pLS76-(<i>LEU2</i>)	p53 R280K	Dr. G. Fronza, Istituto Nazionale per la Ricerca sul Cancro, Italy	Inducible (<i>GAL1-10</i>)
pLS89-(<i>TRP1</i>)	p53 R273H		
	wt p53	Dr. R. Iggo, Swiss Institute for Experimental Cancer Research, Switzerland	
	p53 V122A	Dr. A. Inga, CIBIO, University of Trento, Italy	
pRS314-(<i>TRP1</i>)	TAp73 α		
	TAp63 α		Dr. G. Fronza
	Δ Np63 α		
pTSG-(<i>TRP1</i>)	wt p53	Dr. A. Inga	Inducible (<i>GAL1</i>)
pRB24-(<i>HIS3</i>)	MDM2	Dr. R. Brachmann, Irvine University, USA	Constitutive (<i>PGK1</i>)
ABP140-3xGFP(<i>LEU2</i>)	-	Dr. I. Sagot, Institut de Biochimie et de Génétique Cellulaires, France	Integrative

Table 3. *S. cerevisiae* strains used in yeast assays.

Strain	Genotype	Supplier
CG379	α <i>ade5 his7-2 leu2-112 trp1-289a ura3-52 [Kil-O]</i>	Yeast Genetic Stock Center, University of California, USA
W303	α <i>ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1 can1-100</i>	
W303 <i>atg1</i> Δ ::KanMX4		
W303 <i>atg5</i> Δ ::KanMX4		Dr. D. J. Klionsky, University of Michigan, USA
yLFM-PUMA, RFM-M2	Δ <i>pdr5 [MATa his-, leu2, trp1, ura3, ade2::cyc1-LUC, pdr5::cyc1-REN]</i>	Dr. S. Manon, Institut de Biochimie et de Génétique Cellulaires, CNRS, France
BY4704	<i>MATa ade2::hisG; Dhis3-200; leu2-D0; lys2-D0; met15-D0; trp1-D63</i>	Dr. A. Inga
yLFM-ICORE	<i>MATa ade2-1 leu2-3, 112 trp1-1 his3-11, 15 can1-100 URA3::3XRGC::p-cyc1::ADE2::ura3-1</i>	Euroscarf, Germany
		Dr. A. Inga

2.2.3. Yeast transformation

The plasmids used in the yeast transformation process were first amplified in *Escherichia coli* DH5 α from Lucigen (Frilabo, Porto, Portugal) and thereafter extracted using the GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich, Sintra, Portugal). After extraction, yeast strains were transformed using the LiAc/SS Carrier DNA/PEG method as described in (Gietz and Schiestl, 2007).

For selection of transformed yeast, cells were routinely grown in selective minimal medium with 2% (w/w) glucose (Sigma-Aldrich, Sintra, Portugal), 0.7% (w/w) yeast nitrogen base without amino acids from Difco (Quilaban, Sintra, Portugal) and all the amino acids required for yeast growth (50 μ g/mL) except leucine/tryptophan (for single expression systems) or except leucine and tryptophan (for co-expression systems), and incubated at 30 °C, under continuous orbital shaking (200 r.p.m.).

2.2.4. Growth conditions of transformed yeast cells

For expression of human proteins, cells were diluted to 0.05 optical density at 600 nm (OD₆₀₀) in induction selective medium with 2% (w/w) galactose (Sigma-Aldrich, Sintra, Portugal), 2% (w/w) raffinose (Acros Organics; JMGS, Odivelas, Portugal) (instead of glucose) and 1% glycerol (Sigma-Aldrich, Sintra, Portugal), and incubated at 30 °C, under continuous orbital shaking (200 r.p.m.). In particular, for the dual-luciferase yeast p53 transactivation assay (2.2.10), cells were routinely grown in minimal selective medium with all the amino acids required for yeast growth (50 μ g/mL) except tryptophan (for single expression system) or except tryptophan and histidine (for co-expression system). To induce expression of the human protein, yeast cultures were diluted to approximately 0.1 OD₆₀₀ in selective induction medium containing 0.032 - 0.048% (w/w) galactose (Sigma-Aldrich, Milan, Italy) and 2% (w/w) raffinose (Sigma-Aldrich, Milan, Italy).

Yeast growth was analysed by counting the number of colony-forming units (CFU) per mL (CFU/mL) after 2 days incubation at 30 °C on Sabouraud Dextrose Agar (SDA) from Liofilchem (Frilabo, Porto, Portugal).

2.2.5. Effect of compounds on yeast cell growth

Yeast cells expressing wt p53 were incubated in selective induction medium in the presence of 20 μ M PFT- α , or 0.1% DMSO only, until 0.40 OD₆₀₀ achieved with the control yeast (transformed with the empty vector incubated with DMSO only). Yeast cells

expressing mutant p53 R280K were incubated in selective induction medium in the presence 10 μM α -mangostin (**15**), 10 μM gambogic acid (**16**), or 0.1% DMSO only, until 0.40 OD₆₀₀ achieved with the control yeast (transformed with the empty vector incubated with DMSO only).

Co-transformed yeast cells were incubated in selective induction medium in the presence of 0.1- 50 μM nutlin-3a, 0.1 - 100 μM SJ-172550, 1-100 μM xanthone derivatives **1-12** and **LEM2**, 1-100 μM α -mangostin (**15**), 1-100 μM gambogic acid (**16**), or 0.1% DMSO only, until 0.40 OD₆₀₀ achieved with control yeast (transformed with the empty vectors incubated with DMSO only). Yeast cell growth was analysed as described in **2.2.4**.

2.2.6. Yeast cell cycle analysis

Flow cytometric analysis of DNA content was performed using Sytox Green Nucleic Acid, as described (Coutinho et al., 2009). Briefly, 1×10^7 cells were fixed in 70% (v/v) ethanol, incubated with 250 $\mu\text{g}/\text{mL}$ RNase A (Sigma-Aldrich, Sintra, Portugal) and 1 mg/mL proteinase K (Sigma-Aldrich, Sintra, Portugal), and further incubated with 10 μM Sytox Green Nucleic Acid from Invitrogen (Alfagene, Carcavelos, Portugal).

2.2.7. Yeast cell death assays

To assess the percentage of dead cells, transformed yeasts cells were grown in induction selective medium until 0.4 OD₆₀₀. The number of cells was determined using the clonogenic assay, in which 100 cells were counted and plated on SDA. The number of CFU was determined after 2 days incubation at 30 °C. Results were presented considered 100% survival, the number of CFU obtained with control yeast.

To check for necrotic cell markers, the plasma membrane integrity was monitored by incubation of yeast cells with 5 $\mu\text{g}/\text{mL}$ of propidium iodide (PI) from Molecular Probes (Alfagene, Carcavelos, Portugal) for 10 min at room temperature, followed by flow cytometric analysis. To check for apoptotic markers, the DNA fragmentation was monitored by TUNEL assay performed with *In Situ Cell Death Detection Kit, Fluorescein* (Roche Applied Science), as previously described (Saraiva, et al., 2006). Briefly, yeast cells were fixed with 3.7% (v/v) formaldehyde for 30 min at room temperature and the cell wall was digested with 10 U/mL lyticase for 20 min. After digestion, 10 μL of cell suspension was applied to a slide and incubated at 37°C to dry. The slides were rinsed

with phosphate-buffered saline (PBS) and incubated in permeabilization solution (0.1% triton X-100, 0.1% sodium citrate) for 2 min on ice, rinsed twice with PBS and incubated with 10 μ L of TUNEL reaction mixture for 1 h at 37°C. Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) from Vector Laboratories, Inc (Reagente 5, Porto, Portugal) was used for nuclear staining. Samples were then analysed by fluorescence microscopy.

For the analysis of hydrogen peroxide (H₂O₂)- and acetic acid-induced apoptosis and rapamycin-induced autophagy, yeast cells were grown in induction selective medium until 0.4 OD₆₀₀, followed by treatment with 1.5 and 5 mM H₂O₂ or 100 and 120 mM acetic acid for 1 h or with 0.2 μ g/mL rapamycin for 4 h at 30 °C under continuous shaking. The number of cells was counted and 100 cells were plated on SDA. The number of CFU was determined after 2 days incubation at 30 °C. Results were presented considered 100% survival (0% death) the number of CFU obtained with transformed yeast incubated in the absence of the cell death inducer.

2.2.8. Assessment of reactive oxygen species (ROS) production

To assess intracellular ROS generation, cells were incubated with 5 μ M of CellROX Green Reagent (Life Technologies; Alfacene, Carcavelos, Portugal) for 30 min at 30 °C. Sample analysis was performed by flow cytometry.

2.2.9. Immunofluorescence assays

Immunofluorescence assays were performed as previously described (Coutinho et al., 2011). After fixation and permeabilization, spheroplasts were incubated with anti-p53, anti-p63, or anti-p73 (Table 4) for 2 h, followed by incubation with Alexa Fluor 488 secondary antibody (Table 4) for 2 h. Mounting medium containing 1.5 μ g/mL DAPI from Vector Laboratories, Inc (Reagente 5, Porto, Portugal) was used to visualize nuclear DNA. Samples were analysed by fluorescence microscopy.

2.2.10. Analysis of actin depolarization

Yeast actin organization was analysed using yeast expressing wt p53, TAp63 α , TAp73 α or Δ Np63 α and control yeast (transformed with the empty vector) co-transformed

with the yeast integrative ABP140-3xGFP-(*LEU2*) plasmid (Table 2). Actin organization of co-transformed yeast cells was analysed by fluorescence microscopy.

2.2.11. Dual-luciferase yeast p53 transactivation assay

In this assay, since the expression of luciferase occurs in a p53-dependent manner, the p53 transcriptional activity is reflected by the luciferase activity. Dual-luciferase yeast p53 transactivation assay was performed as previously described (Andreotti et al., 2011). Briefly, a diploid yeast reporter strain was constructed by mating the strain yLFM-PUMA, RFM-M2 with the BY4704 strain. Yeast cells, diluted in selective induction medium as referred in (2.2.4) were transferred to 96-well plates (120 μ L/well) and incubated in the presence of 10 μ M nutlin-3a, 0.6, 2.5 and 10 μ M PFT- α , 10 μ M xanthone derivatives or 0.1% DMSO only, for 16 h (time required by yeast cells to achieve approximately 0.5 OD₆₀₀) at 30 °C, under continuous orbital shaking (150 r.p.m.). To a white 384-well plate were transferred 10 μ L of cell cultures followed by 10 μ L of 2X Passive Lysis Buffer (Promega, Milan, Italy), and 15 min incubation at room temperature with continuous orbital shaking (500 r.p.m.). Afterward, 10 μ L of *Firefly* Luciferase Bright Glo substrate (Promega, Milan, Italy) were added to the cell suspension and the light units were measured with a luminometer (Infinite M-200, Tecan, Italy). To measure *Renilla* activity, 5 μ L of *Firefly* Luciferase Substrate (Luciferase Assay Reagent, LARII) from Promega (Milan, Italy), followed by 5 μ L of the Stop&Glow buffer (Promega, Milan, Italy) were used.

For the analysis of *ACT1* transcription levels, the wt and mutant putative p53 RE mapped at the *ACT1* upstream region was cloned in the yLFM-ICORE reporter strain using an oligonucleotide targeting approach, as previously described (Andreotti et al., 2011). Correct integration of the desired sequence was checked by colony PCR followed by DNA sequencing. The reporter strains were transformed with wt p53 and mutant p53R280K expression vectors (Table 2), and the p53-dependent transactivation of the reporters was examined as described above.

2.2.12. Quantification of mRNA levels

Actin mRNA levels in control yeast and yeast expressing wt p53 was determined by Quantitative PCR (Q-PCR). Total RNA was extracted from yeast, after cell lysis with 0.4-0.6 mm acid washed glass beads (Sigma-Aldrich, Milan, Italy), using the RNeasy

protocol (Qiagen, Milan, Italy). RNA preparations were quantified using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA contaminations were further removed by 15 min incubation with DNase I from New England Biolabs (EuroClone, Milan, Italy) at room temperature, enzyme inactivation at 65 °C for 10 min followed by cDNA synthesis, which was achieved using the RevertAid M-MuLV First Strand cDNA Synthesis Kit (Fermentas, Milan, Italy), starting with 1 µg of total RNA, random hexamers and according to the manufacturer's protocol. The cDNAs were then diluted and 25 ng were used for Q-PCR.

Reactions were developed using the KAPA Sybergreen FAST qPCR MasterMix (Kapa Biosystems, Rome, Italy) in technical triplicates for each biological replicate using the CFX384 real-time PCR detection system (BioRad, Milan, Italy). *ALG9* and *TFC1* were used as reference genes. Primers were selected using the Primer-BLAST online tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and tested for efficiency using standard curves. To verify the complete removal of DNA contamination, a control Q-PCR was performed using as a template mock cDNA preparations obtained omitting the reverse transcriptase enzyme. Relative mRNA quantification was obtained using the $\Delta\Delta C_t$ method (Pfaffl, 2001).

For the of polysomal mRNA levels, the polysome-associated mRNAs were obtained from yeast cells expressing wt p53 and control yeast using the protocol described in (Arava, 2003). Briefly, yeast transformants were grown in 50 mL of selective medium with 0.128% galactose to induce p53 expression and allowed to reach the mid-log phase. Yeast pellets were then processed as indicated in (Arava, 2003). RNA from polysomal fractions was extracted using the phenol/chloroform acid standard procedure as described in (Arava, 2003). The cDNA synthesis and the Q-PCR were performed as referred above.

2.2.13. Fluorescence microscopy

For fluorescence microscopy examination, samples were observed under an Eclipse E400 fluorescence microscope (Nikon, Japan). Yeast cells were observed with an oil immersion lens (PlanFluor 100/1.30), and images were captured by a Digital Sight camera system (Nikon DS-5Mc, Japan) carrying built-in software for image acquisition (Nikon ACT-2U, Japan).

2.3. HUMAN TUMOUR CELL LINES

2.3.1. Human tumour cell lines and growth conditions

The human breast adenocarcinoma-derived MCF-7 cell line was obtained from the InterLab Cell Line Collection, ICLC (Genoa, Italy). The human colon adenocarcinoma HCT116 cell line harboring a wt p53 form (HCT116 p53^{+/+}) and its isogenic derivative, in which p53 has been knocked out (HCT116 p53^{-/-}) were kindly provided by Dr. A. Inga (University of Trento, Italy).

Cell lines were routinely cultured in RPMI with ultraglutamine medium from Lonza (VWR, Carnaxide, Portugal) supplemented with 10% fetal bovine serum from Gibco (Alfagene, Carcavelos, Portugal) and maintained in a humidified incubator at 37 °C with 5% CO₂ in air.

2.3.2. Effect of compounds on in vitro cell growth

Cells were plated in 96-well plates at a final density of 5.0×10^4 cells/well and incubated for 24 h. Cells were then exposed to serial dilutions of each compound (from 0.12 to 50 µM). The effect of the compounds was analysed following 48 h incubation, using the sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006). Briefly, following fixation with 10% trichloroacetic acid (Sigma-Aldrich, Sintra, Portugal), plates were stained with 0.4% SRB (Sigma-Aldrich, Sintra, Portugal) and washed with 1% acetic acid (Sigma-Aldrich, Sintra, Portugal). The bound dye was then solubilized with 10 mL Tris Base and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc., Synergy MX, USA). The solvent of the compounds (DMSO) corresponding to the maximum concentration used in these assays (0.25%) was included as control. The concentration of compound that causes a 50% reduction in the net protein increase in cells (GI₅₀, growth inhibition of 50%) was determined for all tested compounds.

2.3.3. Dual-luciferase reporter assay

Dual-luciferase reporter assay in human tumour cell lines was performed basically as reported in (Bisio et al., 2010). Briefly, 5×10^4 cells/well were plated in 24-well plates and incubated for 24 h. Cells were then transfected at approximately 80% confluence using the Myrus LT-1 reagent (Tema Ricerca, Milan, Italy), and according to the manufacturer's instructions. Specifically, 350 ng of pG13-luc reporter vector was used

along with 50 ng of control pRLSV40 plasmid introduced to normalize the transfection efficiency. After transfection, cells were treated with 1.5 μ M doxorubicin, 10 μ M nutlin-3a, 10 μ M xanthone derivatives or DMSO only, for 16 h. Cells were harvested and the luciferase assay was carried out using the dual-luciferase reagent as described in **2.2.11**.

2.3.4. Analysis of cell cycle

Human tumour cell lines were plated in 6-well plates at a final density of 1.5×10^5 cells/well and incubated for 24 h. Cells were then treated with the GI_{50} concentration of compounds or DMSO only for 48 h. For cell cycle analysis, cells were fixed in ice-cold 70% ethanol and incubated at 37 °C with RNase A (Sigma-Aldrich, Sintra, Portugal) at final concentration of 20 μ g/mL for 15 min, and further incubated with 50 μ g/mL propidium iodide (PI) from Molecular Probes (Alfagene, Carcavelos, Portugal) for 30 min, followed by flow cytometric analysis.

2.4. YEAST AND HUMAN TUMOUR CELLS

2.4.1. Western blot analysis

To obtain protein extracts from yeast, these cells were lysed with CellyticTM Y Cell Lysis Reagent (Sigma-Aldrich, Sintra, Portugal) in the presence of EDTA-free protease inhibitor cocktail (Sigma-Aldrich, Sintra, Portugal). To obtain protein extracts from human tumour cell lines, 1.5×10^5 cells/well were transferred to 6 well-plates and then incubated with compounds for 4, 8 and 16 h. Whole cell lysates were then prepared by lysing the cells with RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Following whole protein quantification using the Bio-Rad Protein Assay (Bio-Rad, Lisbon, Portugal), 50 μ g proteins were electrophoresed on 10-12% SDS-PAGE and transferred to an Amersham nitrocellulose membrane from GE Healthcare (VWR, Carnaxide, Portugal). Membranes were blocked with 5% milk and probed with the primary antibodies presented in Table 4.

After incubation with primary antibodies, membranes were probed with an anti-mouse or anti-rabbit horseradish-peroxidase (HRP)-conjugated secondary antibody. For loading control, membranes were stripped and reprobed with an anti-yeast phosphoglycerate kinase (Pgk1p) antibody for yeast, or with an anti-actin antibody for tumour cell lines. The signal was detected with the ECL Amersham kit from GE

Healthcare (VWR, Carnaxide, Portugal) and the Kodak GBX developer and fixer (Sigma-Aldrich, Sintra, Portugal). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat, Marne La Vallée, France) and were normalized against the control sample, which was set as 1.

2.4.2. Analysis of phosphatidylserine exposure

The analysis of phosphatidylserine exposure in yeast and human tumour cells was performed by Annexin V assay using the Annexin V: FITC Apoptosis Detection Kit I (BD Biosciences, Enzifarma, Porto, Portugal), according to the manufacturer's instructions. For yeast, cell wall was digested as described in **2.2.7**. Human tumour cells were plated in 6-well plates at a final density of 1.5×10^5 cells/well. After 24 h incubation, tumour cells were treated with the GI_{50} of LEM2 or DMSO only for 48 h. Yeast and human tumour cells were then resuspended in binding buffer and then incubated with 5 μ L of FITC-Annexin V and 5 μ L of PI for 15 min at room temperature. Cells were then analysed by flow cytometry.

2.4.3. Flow cytometric data acquisition and analysis

For the flow cytometric analysis the FACSCalibur flow cytometer from BD Biosciences (Enzifarma, Porto, Portugal) and the CellQuest software from BD Biosciences (Enzifarma, Porto, Portugal) were used. Yeast cell cycle phases were identified and quantified using ModFit LT software (Verity Software House Inc., Topsham, USA).

2.4.4. Statistical analysis

Statistical analyses were performed using SigmaStat 3.5 programme. Unpaired Student's *t*-test and two-way ANOVA ($P < 0.05$) were used to evaluate the statistical significance of the differences between means.

Table 4. Antibodies used in Western blot.

Antigen	Final Dilution	Supplier
Primary antibodies		
p53 (DO-1) (mouse monoclonal)	1:500 (yeast) 1:8000 (human cells) 1:200 (Immunofluorescence assay)	Santa Cruz Biotechnology (Frilabo, Porto, Portugal)
MDM2 (SMP14) (mouse monoclonal)	1:200	
Caspase-7 (B4-G2) (mouse monoclonal) ¹	1:500	
Bax (2D2) (mouse monoclonal)	1:1000	
p21 (C-19) (rabbit polyclonal)	1:2000	
Actin (C-11) (rabbit polyclonal)	1:500 (yeast) 1:8000 (human cells)	
p63 (4A4) (mouse monoclonal) ²	1:500 1:200 (Immunofluorescence assay)	
Atg8p (Aut7; γ -117) (rabbit polyclonal)	1:200	
PARP (C2-10) (mouse monoclonal)	1:1000	
MDMX (A300-287A) (rabbit polyclonal)	1:2000	Bethyl Laboratories
p73 (AB7824) (rabbit polyclonal)	1:1000 1:100 (Immunofluorescence assay)	Millipore (Grupo Taper, Sintra, Portugal)
Pgk1p (22C5D8) (mouse monoclonal)	1:5000	Molecular Probes (Alfagene, Carcavelos, Portugal)
Secondary antibodies		
Anti-mouse horseradish- peroxidase (HRP)- conjugated	1:5000	Santa Cruz Biotechnology (Frilabo, Porto, Portugal)
Anti-rabbit horseradish- peroxidase (HRP)- conjugated	1:5000	

¹ Detects the caspase-7 full length precursor and the large subunit (LS) of cleaved caspase-7.

² Detects TAp63 α and Δ Np63 α .

2.5. COMPUTATIONAL CHEMISTRY: DOCKING OF A LIBRARY OF XANTHONE DERIVATIVES IN MDM2

To prepare a library of virtual xanthenes and known inhibitors of the p53-MDM2 interaction, 58 xanthone derivatives described in (Pedro et al., 2002; Sousa et al., 2002; Castanheiro et al., 2007; Marquez-Valadez et al., 2009; Sousa et al., 2009; Costa et al., 2010; Palmeira et al., 2010; Palmeira et al., 2011; Paiva et al., 2012), two known inhibitors

of the p53-MDM2 interaction (Vassilev, 2007), and several known non-ligands (Clement et al., 2008; Datta et al., 2010) were drawn (ChemSketch, ACD/Labs 2007) and subjected to energy minimization (ArgusLab version 4.0.1 for Windows) by molecular mechanics using the force field method (Kini et al., 1991). Docking simulations in MDM2 (pdb code: 1YCR) were undertaken in AutoDock Vina (Trott et al., 2010). AutoDock Vina considered the target conformation as a rigid unit, while the ligands were allowed to be flexible and adaptable to the target. A search exhaustiveness of 8 was employed. The 9 lowest energy conformations for each ligand were retrieved. Chimera 1.6.1 (Pettersen et al., 2004) and Pymol 0.99 (Seeliger et al., 2010) were used for visual inspection of results and graphical representations.

Chapter 3

Results and Discussion

Chapter 3.1.

Discovery of a new small molecule inhibitor of the p53-MDM2 interaction using a yeast-based approach

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Xanthone derivatives have been widely reported as potential anticancer agents [reviewed in (Pinto et al., 2005; Pouli and Marakos, 2009)]. In fact, in the last years, molecular modifications on the tricyclic xanthone scaffold led to potent inhibitors of the growth of several human tumour cell lines (Pedro et al., 2002; Sousa et al., 2002; Castanheiro et al., 2007; Sousa et al., 2009; Palmeira et al., 2011). Particularly, prenylated xanthone derivatives have drawn attention due to their potency and selectivity against breast adenocarcinoma MCF-7 tumour cells with wt p53 (Castanheiro et al., 2007).

In the present work, with the virtual screening of a library of xanthone derivatives, potential novel MDM2 ligands were identified, and their activities as inhibitors of the p53-MDM2 interaction were investigated using yeast cell-based screening assays. Using this approach, the pyranoxanthone 3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one (**1**; Fig. 13), was identified as a promising inhibitor of the p53-MDM2 interaction, which successfully activated p53 and downstream cell signalling in human tumour cells. With our findings, a new small molecule inhibitor of the p53-MDM2 interaction with a xanthone scaffold was identified for the first time.

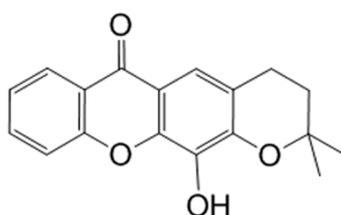


Figure 13. Chemical structure of 3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one (pyranoxanthone 1).

3.1.1. Development of a yeast phenotypic assay for the screening of inhibitors of the p53-MDM2 interaction

A previous work performed by our group showed that expression of human wt p53 in *S. cerevisiae* induced growth inhibition associated with S-phase cell cycle arrest (Coutinho et al, 2009b). Here, it is shown that although the single expression of human MDM2 in yeast did not interfere with the cell growth (therefore represented by the control yeast, transformed with the empty vectors and treated with DMSO only, in Figures 14B and 15A) and cell cycle progression, its co-expression with wt p53 significantly reduced the p53-induced growth inhibition and cell cycle arrest (Fig. 14A–C). These results

strengthens the previously reported conservation in yeast of the negative effect of MDM2 on p53 activity (Oliner et al., 1993; Di Ventura et al., 2008; Andreotti et al., 2011), and gave rise to a possible use of these cells in the development of a simple growth-inhibition screening assay to search for inhibitors of the p53-MDM2 interaction. In this yeast assay, inhibitors of the p53-MDM2 interaction would abolish or abate the negative effect of MDM2 on p53, restoring the p53-induced growth inhibition and S-phase cell cycle arrest. The assay was, in fact, validated by testing nutlin-3a, the known inhibitor of the p53-MDM2 interaction (Vassilev et al., 2004; Tovar et al., 2006).

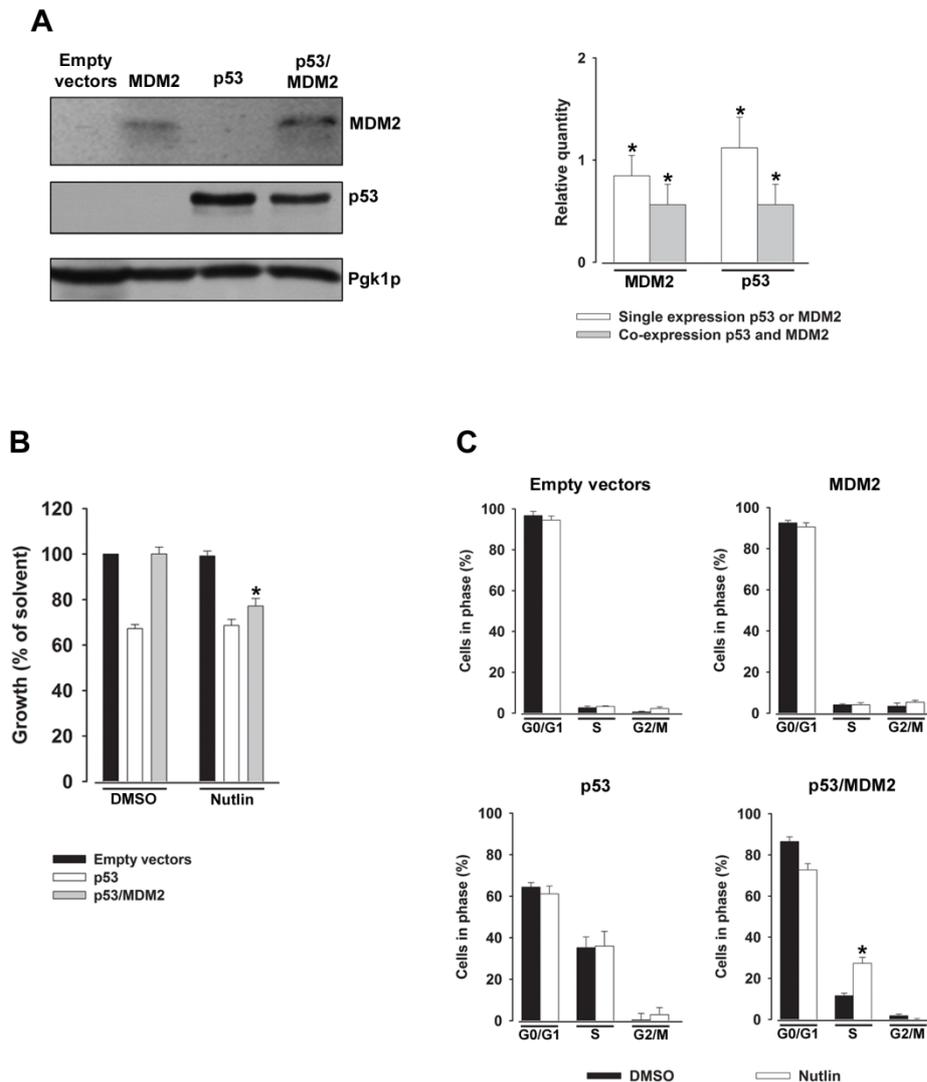


Figure 14. Nutlin-3a reduces the negative effect of MDM2 on p53 activity in yeast. Co-transformed yeast cells were incubated in selective induction medium in the presence of 10 μ M nutlin-3a or DMSO only, for 42 h. **(A)** Expression of human wt p53 and/or human MDM2 in yeast was confirmed by Western blot analysis. Pgk1p was used as loading control. Immunoblots represent one of two independent experiments. In the quantification of MDM2/p53 band intensities, values are mean \pm S.E.M. of two independent experiments; values significantly different from

control yeast (empty vectors): $*P < 0.05$. **(B)** Effects of nutlin-3a on the growth of control yeast, yeast expressing only p53 and yeast co-expressing p53 and MDM2. Results are plotted setting as 100% growth the number of CFU obtained with control yeast. The growth of yeast expressing MDM2 alone treated with nutlin-3a was approximately 100%, and therefore it is represented by the control yeast treated with DMSO only. Data are mean \pm S.E.M. of five independent experiments; values obtained with yeast co-expressing p53 and MDM2 treated with nutlin-3a significantly different from DMSO only ($*P < 0.05$). **(C)** Effects of nutlin-3a on the yeast cell cycle progression of control yeast, yeast expressing only MDM2 or p53 and yeast co-expressing p53 and MDM2. Yeast cell cycle phases were quantified by flow cytometry; data are mean \pm S.E.M. of two independent experiments; values significantly different from DMSO only ($*P < 0.05$).

Dose-response curves for the effects of 0.1–50 μ M nutlin-3a on the reduction of the MDM2 negative effect on p53-induced growth inhibition in yeast co-expressing p53 and MDM2 and on the inhibition of growth of control yeast were obtained (data not shown). The concentration of 10 μ M nutlin-3a was selected as the lowest concentration for which a significant reduction of the negative effect of MDM2 on p53-induced growth inhibition was obtained without cytotoxic effects on control yeast.

Altogether, with the obtained results a yeast phenotypic assay, based on simple measurements of yeast cell growth and analysis of yeast cell cycle, was developed that may enable us to search for inhibitors of the p53-MDM2 interaction.

3.1.2. Virtual screening of a library of xanthone derivatives to search for potential MDM2 ligands

The virtual screening of a library of xanthone derivatives resulted in a list of potential MDM2 ligands ranked according to their binding affinities. Docking simulations in PyRx/AutoDock Vina produced nine docked conformations for each ligand. The total number of compounds screened was 60. These small molecules correspond mainly to xanthone derivatives with diverse substituents, such as simple oxygenated, prenylated, lignoids, and others (Pedro et al., 2002; Sousa et al., 2002; Castanheiro et al., 2007; Sousa et al., 2009; Costa et al., 2010; Palmeira et al., 2010; Palmeira et al., 2011; Paiva et al., 2012;). Two known inhibitors of the p53-MDM2 interaction, nutlin-3a, and (4-chlorophenyl)[3-(4-chlorophenyl)-7-iodo-2,5-dioxo-1,2,3,5-tetrahydro-4H-1,4-benzodiazepin-4-yl]acetic acid (bzd) were used in the docking studies as positive controls along with known non-ligands of MDM2, used as negative controls.

MDM2 residues *Gly58*, *Asp68*, *Val75*, and *Cys77* are critical for the interaction with p53 (Freedman et al., 1997). In the virtual screening against MDM2 (PDB code: 1YCR), to study only the interactions established by a ligand in this binding site, the grid box was placed in a position so that the ligand could not interact elsewhere with the MDM2 (see Appendix; Fig. A1). The binding affinity values found for the best scoring conformations of the well-characterized inhibitors of the p53-MDM2 interaction, which are known to establish interactions with MDM2 protein, were between -6.9 (bzd) and -5.6 (nutlin-3a) kcal.mol⁻¹. A total of 46 xanthone derivatives showed binding affinity values in this range (see Appendix; Table A1), with the pyranoxanthone **1** revealing the highest binding affinity (-7.6 kcal.mol⁻¹). However, limiting the docking studies to the above mentioned grid, the nutlin-3a showed a binding affinity lower than the expected (Warner et al., 2012). Nutlin-3a was previously predicted to contact with nine MDM2 amino acids (Warner et al., 2012) and, by limiting the interaction site using the grid box, nutlin-3a was not allowed to contact with all the MDM2 amino acids. Subsequently, the study of the interactions established by the ligands with the entire MDM2 protein (1YCR) was performed removing the grid box. In this case the absolute binding affinity value of nutlin-3a rose to -7.4 kcal.mol⁻¹ binding at a different site in the MDM2 protein (see Appendix; Table A2). However, the binding affinity of the hit pyranoxanthone **1** was the same (-7.6 kcal.mol⁻¹). A possible explanation for these results is the inexistence of other interaction sites beyond the 4 amino acids selected with the grid box in the MDM2 protein that would lead to higher pyranoxanthone **1** binding affinity.

Based on the overall *in silico* results and on the IC₅₀ (concentration required to cause 50% growth inhibition) values against human tumour cells with wt p53 (Pedro et al., 2002; Sousa et al., 2002; Costa et al., 2010; Sousa et al., 2011; Paiva et al., 2012), the xanthone derivatives **1-14** were selected for the yeast cell-based screening assay (Table 5; xanthenes **13** and **14** were not tested due to the low solubility in DMSO).

Table 5. Effects of compounds on the inhibitory activity of MDM2 on p53-induced yeast growth inhibition.

Compounds	Growth Inhibition (% of p53 effect)
DMSO	0.2 ± 4.2
Nutlin-3a	67.8 ± 3.3
1	55.8 ± 5.5
2	0.12 ± 1.06
3	20.1 ± 5.6
4	-0.8 ± 9.6
5	7.06 ± 9.1
6	-5.29 ± 1.2
7	-7.71 ± 2.0
8	-5.80 ± 1.5
9	-8.61 ± 2.0
10	<i>ND</i>
11	<i>ND</i>
12	<i>ND</i>

Yeast cells co-expressing p53 and MDM2 were incubated in galactose selective medium in the presence of 10 μ M compound or DMSO only, for 42 h. Nutlin-3a was used as positive control. The effect obtained with p53 alone was considered 100% growth inhibition. Data represent mean \pm S.E.M. of five independent experiments. *ND*: Not determined (a cytotoxic effect on control yeast was observed).

3.1.3. Identification of pyranoxanthone 1 as a promising inhibitor of the p53-MDM2 interaction using the yeast approach

Using the developed yeast phenotypic assay, the activity of the most promising xanthone derivatives **1–12** on p53-MDM2 interaction was investigated. Since xanthonenes **10–12** were cytotoxic in control yeast, their effects on p53-MDM2 interaction were not evaluated. Among the tested xanthonenes, only the pyranoxanthone **1** significantly reduced the MDM2 inhibitory effect on p53 activity (Table 5; Fig. 15A-B; effects of xanthonenes **2–9** are represented by xanthone **2**), without interfering with the activity of p53 or MDM2 when expressed alone (Fig. 15A, B; in Fig. 15A similar effects of pyranoxanthone **1** were obtained on control yeast and yeast expressing only MDM2). In fact, likewise nutlin-3a, when yeast cells co-expressing p53 and MDM2 were treated with 10 μ M pyranoxanthone **1** for 42 h, approximately 56% and 87% of the p53-induced growth inhibition and S-phase cell cycle arrest, respectively, were re-established (Table 5; Fig. 15A, B). Dose-response curves for the effects of 1–100 μ M pyranoxanthone **1** on the reduction of the MDM2 negative effect on p53-induced growth inhibition in yeast co-expressing p53 and MDM2 and on the inhibition of growth of control yeast were obtained (Fig. 15C). The concentration of 10 μ M pyranoxanthone **1** was selected as the lowest concentration for which a significant reduction of the negative effect of MDM2 on p53-induced growth inhibition was obtained without cytotoxic effects on control yeast. In opposition, xanthonenes **2–9** did not interfere with the negative effect of MDM2 on p53-induced yeast growth inhibition (Table 5; Fig. 15A; represented by xanthone **2**) and cell cycle arrest (Fig. 15B; represented by xanthone **2**). The effect of the xanthone derivatives on the negative regulation of p53-dependent transcriptional activity by MDM2 was also analysed in yeast using a dual-luciferase p53 transactivation assay previously reported in (Andreotti et al., 2011). This assay was carried out in yeast cells co-expressing p53 and MDM2, and used the p53 RE derived from the *BBC3/PUMA* gene fused to a luciferase reporter. The results showed that, likewise to 10 μ M nutlin-3a, 10 μ M pyranoxanthone **1** significantly increased the luciferase activity. This indicated a reversion of the MDM2 inhibitory effect on p53 transcriptional activity by pyranoxanthone **1** (Fig. 15D). As obtained in the yeast phenotypic assay, xanthonenes **2–9** did not interfere with the p53 transcriptional activity (Fig. 15D; represented by xanthone **2**). Altogether, the results obtained in yeast strongly supported that pyranoxanthone **1** was a promising inhibitor of the p53-MDM2 interaction.

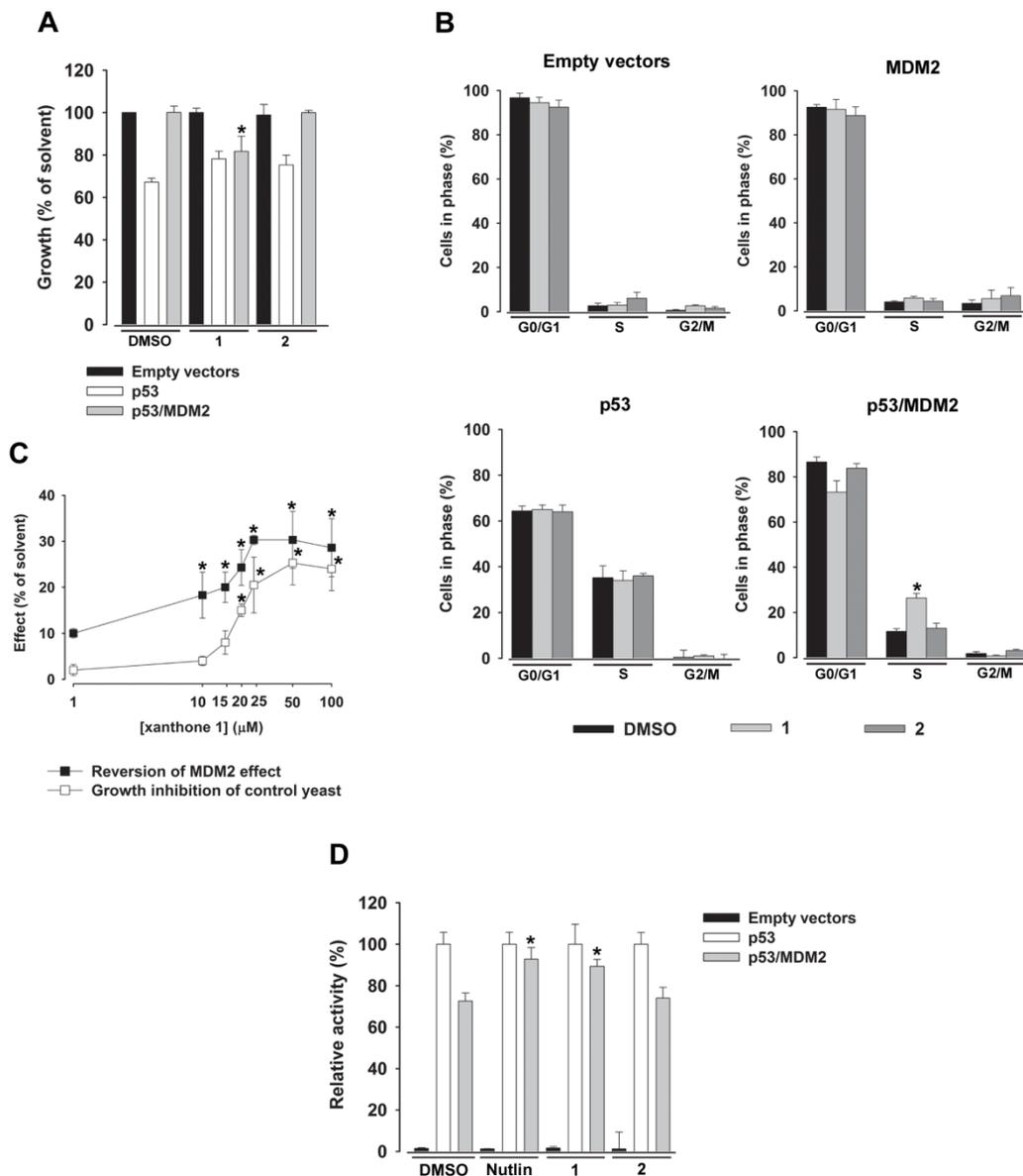


Figure 15. Pyranoxanthone 1 reduces the negative effect of MDM2 on p53 activity in yeast.

Co-transformed yeast cells were incubated in induction selective medium in the presence of 10 μ M xanthenes **1** and **2** (as representative of non-active xanthenes) or DMSO only, for 42 h (in **A - C**) or 16 h (in **D**). (**A**) Effects of xanthenes **1** and **2** on the growth of control yeast (empty vectors), yeast expressing only p53 and yeast co-expressing p53 and MDM2. Results are plotted setting as 100% growth the number of CFU obtained with control yeast treated with DMSO only. The growth of yeast expressing MDM2 alone treated with compounds was approximately 100%, and therefore it is represented by the control yeast. Data are mean \pm S.E.M. of five independent experiments; values obtained with yeast co-expressing p53 and MDM2 treated with compounds significantly different from DMSO only ($*P < 0.05$). (**B**) Effects of xanthenes **1** and **2** on the yeast cell cycle progression of control yeast, yeast expressing only MDM2 or p53 and yeast co-expressing p53 and MDM2. Yeast cell cycle phases were quantified by flow cytometry; data are mean \pm S.E.M. of two independent experiments; values significantly different from DMSO only ($*P < 0.05$). (**C**) Effects of

1-100 μM xanthone **1** on the reduction of MDM2 negative effect on p53-induced growth inhibition in yeast co-expressing p53 and MDM2 and on the growth inhibition of control yeast. Data are mean \pm S.E.M. of four independent experiments; values significantly different from DMSO only ($*P < 0.05$). (D) Effects of xanthenes **1** and **2** on the p53-dependent transcriptional activity in control yeast, yeast expressing only p53 and yeast co-expressing p53 and MDM2. Dual-luciferase yeast p53 transactivation assay was performed with the p53 RE PUMA fused to a luciferase gene. Nutlin-3a was used as positive control. Results are plotted as relative to the transcriptional activity achieved by p53 alone (set to 100%). Presented are mean \pm S.E.M. of four independent experiments; values obtained with yeast co-expressing p53 and MDM2 treated with compounds significantly different from DMSO only ($*P < 0.05$).

3.1.4. Pyranoxanthone 1 reactivates the p53 activity and downstream cell signalling in human tumour cells

The molecular mechanism of action of pyranoxanthone **1** as inhibitor of the p53-MDM2 interaction was further ascertained in human tumour cells harboring wt p53, and was compared to that obtained with known small molecule activators of p53 activity, 1.5 μM doxorubicin and 10 μM nutlin-3a. In order to analyse the effect of pyranoxanthone **1** on p53-dependent transcriptional activity, a p53 reporter assay was performed in human tumour cells with wt p53, HCT116 p53^{+/+} (Fig. 16A) and MCF-7 (Fig. 16C). In parallel, the activity of pyranoxanthone **1** was evaluated in p53 null-HCT116 cells (HCT116 p53^{-/-}; Fig. 16B) and MCF-7 cells transfected with an empty reporter vector, which was used as negative controls (Fig. 16D). In this assay, the synthetic p53 RE pG13 fused to the luciferase reporter gene was used. The obtained results showed that, likewise doxorubicin (in HCT116 p53^{+/+}), and doxorubicin and nutlin-3a (in MCF-7), 10 μM pyranoxanthone **1** markedly increased the luciferase activity in HCT116 p53^{+/+} (Fig. 16A) and MCF-7 (Fig. 4C) tumour cells. On the contrary, it did not interfere with the luciferase activity in p53-negative control cell lines exposed to the same conditions (Fig. 16B and D). These results demonstrated that the pyranoxanthone **1** successfully activated the p53-dependent transcriptional activity in human tumour cell lines.

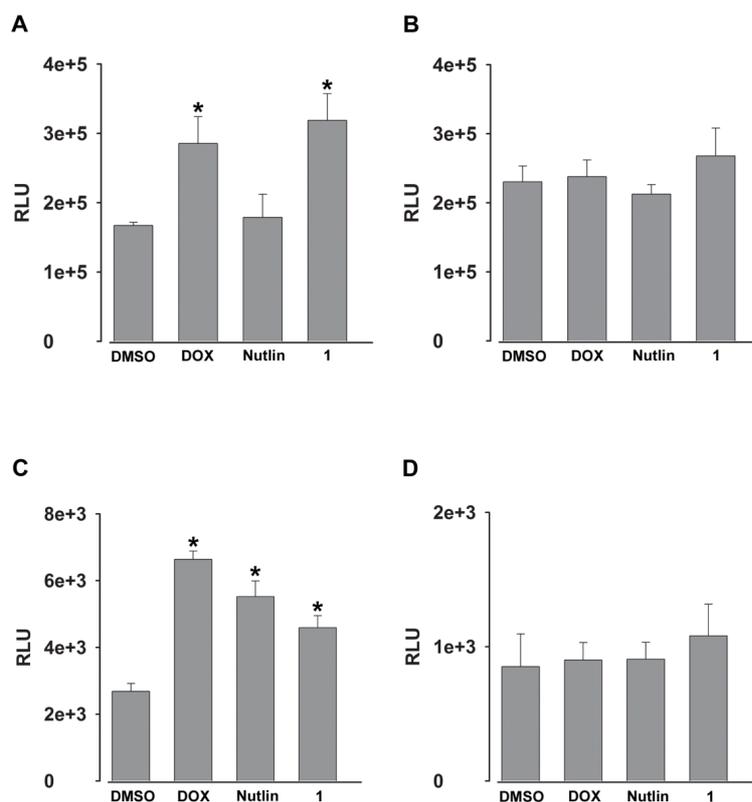


Figure 16. Pyranoxanthone 1 increases the p53-dependent transcriptional activity in human tumour cells. For the dual-luciferase gene reporter assay, the human tumour cells transfected with the pG13-luciferase reporter vector (or the empty reporter vector) were incubated in the presence of 1.5 μ M doxorubicin (DOX), 10 μ M nutlin-3a, 10 μ M pyranoxanthone 1 or DMSO only for 16 h. Doxorubicin and nutlin-3a were used as positive controls. **(A–C)** Effects of pyranoxanthone 1 on the pG13-luciferase reporter activity in: **(A)** HCT116 p53^{+/+}, **(B)** HCT116 p53^{-/-} (negative control of A), and **(C)** MCF-7 cells. In **(D)**, MCF-7 cells were transfected with the empty reporter vector (negative control of C). RLU, relative light units. Data are mean \pm S.E.M. of three independent experiments; values significantly different from DMSO only: * $P < 0.05$.

The effect of pyranoxanthone 1 on the stabilization of p53 protein levels was also analysed in HCT116 tumour cells. As expected, similarly to positive controls, 10 μ M pyranoxanthone 1 increased the p53 baseline levels upon 4, 8 and 16 h treatments in HCT116 p53^{+/+} cells, suggesting an inhibition of the p53 degradation by MDM2 in these cells (Fig. 17A). However, contrarily to nutlin-3a, for the different tested time points, no increase on MDM2 protein levels was observed in these tumour cells after treatment with 10 μ M pyranoxanthone 1 (Fig. 17B). In spite of this, likewise positive controls, 10 μ M pyranoxanthone 1 increased the expression levels of other proteins encoded by p53 target genes, namely p21 (Fig. 17C) and Bax (Fig. 17D) after 16 and 8 h treatments,

respectively, in HCT116 p53^{+/+} but not in HCT116 p53^{-/-} tumour cells. Similarly, it enhanced the procaspase-7 cleavage to the active caspase-7 (LS) form at 8 h treatment in HCT116 p53^{+/+}, but not in HCT116 p53^{-/-} tumour cells (Fig. 17E).

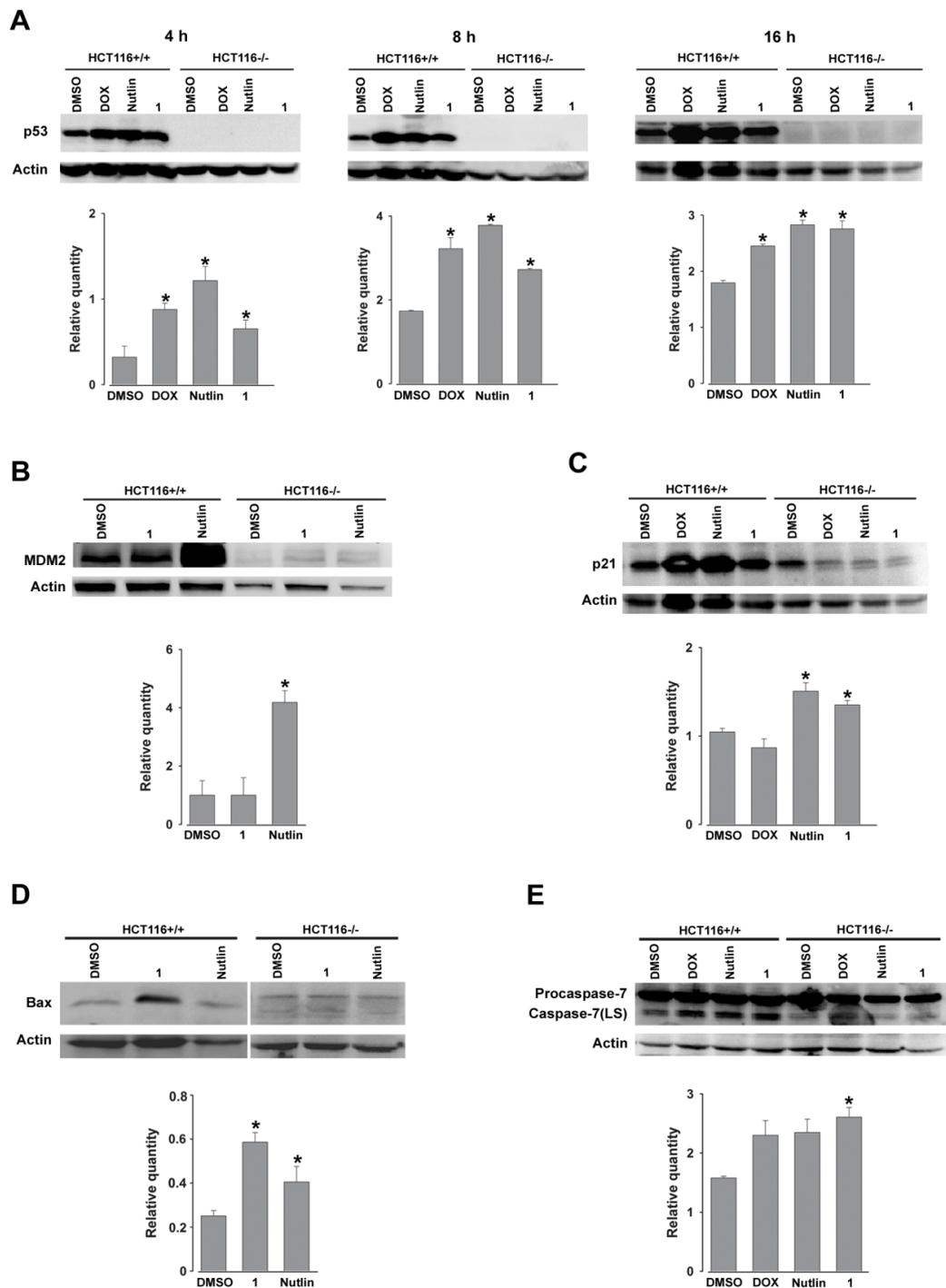


Figure 17. Pyranoxanthone 1 leads to p53 stabilization, increases p21 and Bax levels, and enhances procaspase-7 cleavage to active caspase-7 in HCT116 p53^{+/+} cells. HCT116 tumour cells were incubated in the presence of 1.5 μ M doxorubicin (DOX), 10 μ M nutlin-3a, 10 μ M pyranoxanthone **1** or DMSO only for 4, 8 and 16 h. Doxorubicin and nutlin-3a were used as positive controls. Protein expression levels were analysed by Western blot and normalized to the actin

loading control. Effects of pyranoxanthone **1** on the protein levels of: (A) p53 at 4, 8 and 16 h; (B) MDM2 at 8 h; (C) p21 at 16 h; (D) Bax at 8 h. (E) Pyranoxanthone **1** enhances procaspase-7 cleavage to the active caspase-7 (LS) form at 8 h. In the quantification of band intensities obtained in **A–E**, values are mean \pm S.E.M. of two independent experiments; values significantly different from DMSO only: * $P < 0.05$.

Altogether, these results confirmed the effectiveness of the pyranoxanthone **1** in human tumour cells with wt p53. In fact, the pyranoxanthone **1** activated the p53-dependent transcriptional activity, and increased the protein levels of p53, p21, Bax and cleaved caspase-7.

3.1.5. Analysis of the predicted binding model of pyranoxanthone 1 to MDM2 supports that pyranoxanthone 1 binds to MDM2

Analysis of the predicted binding model of pyranoxanthone **1** to MDM2 supports that pyranoxanthone **1** binds to MDM2. Based on the obtained results, a careful visual inspection of the pyranoxanthone **1** on the limited interaction site of MDM2 was performed by computational docking. As shown in Figure 18A, the best fitting molecule (pyranoxanthone **1**) adopted a pose within the p53-binding site, filling the space supposedly occupied by the p53 helix. Figure 18B highlights the polar interaction established between pyranoxanthone **1** and MDM2. In contrast, nutlin-3a–MDM2 interaction revealed no hydrogen bonding (Fig. 18C). This obtained result is in accordance to (Warner et al., 2012), in which was verified that nutlin-3a binds to MDM2 mainly by hydrophobic interactions, where it is predicted to contact nine MDM2 amino acids, but all without hydrogen bonds.

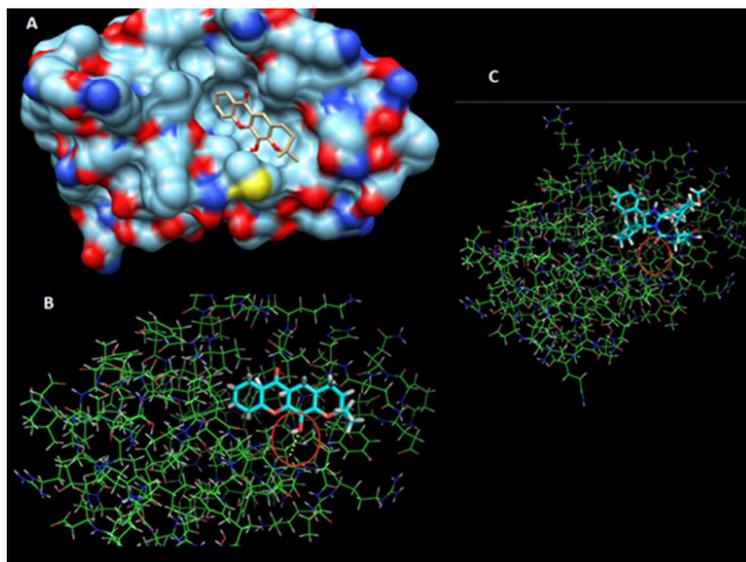


Figure 18. Predicted binding model using computational docking for the hit pyranoxanthone 1. (A) Pyranoxanthone 1 in the binding site of MDM2. (B) Polar interaction (highlighted with the red circle) established by pyranoxanthone 1 (blue) in the MDM2 (green) binding site. (C) Absence of hydrogen bond between MDM2 and nutlin-3a.

3.1.6. Discussion

To search for new inhibitors of the p53-MDM2 interaction several assays have been developed in the last years. However, most of them are actually quite expensive when applied to the screening of large libraries of compounds, limiting their use in drug discovery. In the present work, a simple, selective, and reliable yeast growth inhibition assay was developed to search for novel inhibitors of the p53-MDM2 interaction. This assay can be easily adapted to the HTS of large chemical libraries based on simple measurements of the yeast cell growth in a cost-effective manner [reviewed in (Pereira et al., 2012b)]. Herein, the use of this yeast assay led us to the identification of a potential lead compound (pyranoxanthone 1), thus providing a proof of concept for its effectiveness in the discovery of new inhibitors of the p53-MDM2 interaction. The yeast growth inhibition assay proves to be greatly useful in the initial screening for a first selection of most promising compounds to be tested in more complex cell models.

In this work, using the virtual screening based on multiple binding modes, a set of putative MDM2 ligands with a xanthone scaffold was identified. Based on the generated ranking docking scores, 14 of the potential ligands, exhibiting in our previous studies antiproliferative activity against tumour cells with wt p53 (Sousa et al., 2002; Sousa et al., 2011; Paiva et al., 2012), were selected. The activity of these 14 compounds as inhibitors

of p53-MDM2 interaction was subsequently investigated using the developed yeast phenotypic screening assay followed by a yeast p53 transactivation assay (described in Andreotti et al., 2011). Using this yeast assay, the pyranoxanthone **1** (3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one) was identified as a promising inhibitor of the p53-MDM2 interaction. Interestingly, the active hit identified in yeast also exhibited the highest binding affinity ($-7.6 \text{ kcal mol}^{-1}$) to the MDM2 local of interaction with p53 in the virtual screening. Nevertheless, a correlation between docking scores and activity was not observed and future studies would require considering not only the ligands, but also the target as flexible to improve the accuracy of the docking results.

To further ascertain whether the pyranoxanthone **1** had effects on tumour cells consistent with an inhibition of the p53-MDM2 interaction, the activity of this compound was analysed in human tumour-derived cell lines with wt p53. In a recent work, pyranoxanthone **1** revealed a higher potency against tumour cells with wt p53 (MCF-7; $GI_{50} = 5.3 \pm 0.7$) than against tumour cells with mutant p53 (MDA-MB-231; $GI_{50} = 29.0 \pm 3.6$) (Paiva et al., 2012). Supporting previous results from our group (Sousa et al., 2002; Sousa et al., 2011; Paiva et al., 2012), the present work confirmed the effectiveness of pyranoxanthone **1** in tumour cells with wt p53. Most importantly, herein it is shown that, in conformity to what was obtained in yeast, the pyranoxanthone **1** mimicked the activity of known small molecule activators of p53 activity in human tumour cells, leading to the successful activation of p53 and downstream cell signaling.

By computational docking studies, it was possible to ascertain that similarly to nutlin-3a, pyranoxanthone **1** binds to MDM2, therefore activating p53 through inhibition of MDM2 function in tumour cells. However, distinct types of interactions were detected for these two compounds. While interaction of pyranoxanthone **1** with MDM2 involves hydrogen interaction with *Gly58*, a residue critical for the interaction of MDM2 with p53, nutlin-3a-MDM2 interaction mainly involves hydrophobic interactions.

As a whole, in this work, the pyranoxanthone **1** (3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one) was identified as a new inhibitor of the p53-MDM2 interaction. Our finding thus adds, for the first time, the xanthone scaffold to the list of chemotypes of small molecule inhibitors of the p53-MDM2 interaction. In contrast to nutlin-3a, the pyranoxanthone **1** represents a very promising achiral compound with feasible synthesis and with different predicted binding modes to MDM2. Besides its potential use as molecular probe and possible anticancer agent, pyranoxanthone **1** mainly represents a useful lead compound for the structure-based design of more potent drug-like analogs.

Chapter 3.2.

α -Mangostin and gambogic acid as potential inhibitors of the p53–MDM2 interaction revealed by a yeast approach

Leão M, Gomes S, Pedraza-Chaverri J, Machado N, Sousa E, Pinto M, Inga A, Pereira C, Saraiva L.

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α -Mangostin (**15**) and gambogic acid (**16**) (Fig. 19) are prenylated xanthenes naturally obtained from the mangosteen fruit of *Garcinia mangostana* L. (Clusiaceae) and from the resin of *Garcinia hanburyi* Hook.f. (Clusiaceae), respectively. These natural products are well-known for their potent cytotoxic activity against several human tumour cell lines [reviewed in (Pedraza-Chaverri et al., 2008; Chantarasriwong et al., 2010)] and also for their antitumor activity in animals bearing tumours (Zhao et al., 2008; Chitchumroonchokchai et al., 2013).

In a previous study, a new inhibitor of the p53-MDM2 with a xanthone scaffold was identified. Based on this finding, it was hypothesized that α -mangostin (**15**) and gambogic acid (**16**) may affect the p53-MDM2 interaction.

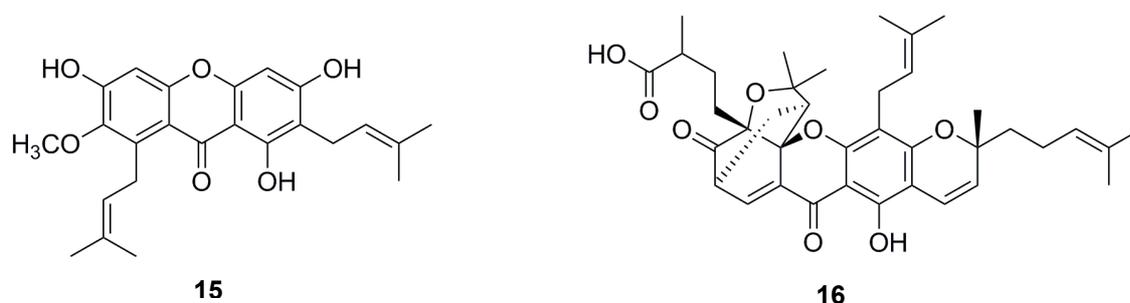


Figure 19. Chemical structure of α -mangostin (15**) and gambogic acid (**16**).**

3.2.1. Identification of α -mangostin and gambogic acid as potential inhibitors of the p53-MDM2 interaction using the yeast approach

To address this issue, the previously developed yeast-based assays to search for inhibitors of the p53-MDM2 interaction were used. In the yeast phenotypic assay used, although the expression of human MDM2 did not interfere with cell growth and cell cycle progression, its co-expression with wt p53 significantly reduced the p53-dependent growth inhibition and cell cycle arrest (Fig. 20A, B). Inhibitors of the p53-MDM2 interaction, such as the positive control nutlin-3a, abolish or markedly reduce the negative impact of MDM2 on p53 activity, thus restoring the p53-induced growth inhibition and cell cycle arrest. Notably, these small molecules do not interfere with the activity of p53 or MDM2 when expressed alone (Fig. 20A, B; in A, the effect of nutlin-3a on yeast cells expressing only MDM2 was similar to that obtained with control yeast, transformed with the empty vectors). Using the yeast growth-inhibition assay, the effect of 1-100 μ M α -mangostin (**15**) and gambogic acid (**16**) was evaluated using as end points the MDM2-dependent

inhibition of p53-induced growth inhibition (Fig. 20C). From the concentration-response curves obtained, a concentration of 10 μM was selected for both xanthenes as the lowest concentration at which a significant reversion of the MDM2 effect was obtained. Likewise, 10 μM of nutlin-3a, α -mangostin (**15**), and gambogic acid (**16**) reverted the inhibitory effect of MDM2 on p53-induced growth inhibition and S-phase cell cycle arrest, without interfering with the activity of p53 or MDM2 when expressed alone (Fig. 20A, B; in A, the effect of xanthenes **15** and **16** on yeast cells expressing only MDM2 was similar to that obtained with the control yeast, transformed with the empty vectors).

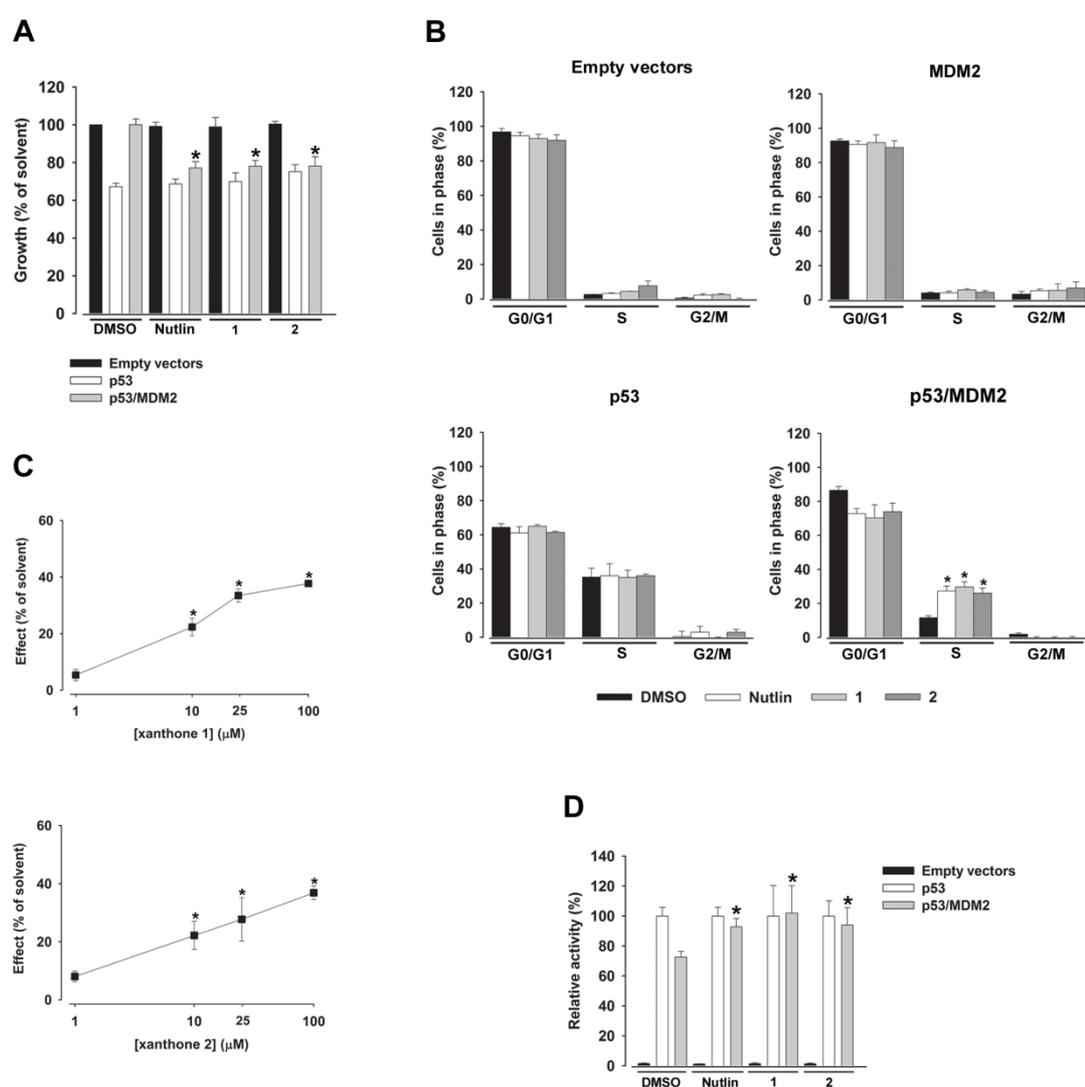


Figure 20. α -Mangostin (**15**) and gambogic acid (**16**) revert the inhibitory effect of MDM2 on p53-induced growth inhibition and S-phase cell cycle arrest, as well as on p53-dependent transcriptional activity in yeast. Yeast cells were incubated in the presence of 10 μM nutlin-3a, 1–100 μM xanthenes **15** and **16**, or DMSO only, for 42 h (A–C) or 16 h (D). Nutlin-3a was used as positive control. (A) Effects of 10 μM nutlin-3a and 10 μM xanthenes **15** and **16** on the growth of

control yeast, yeast expressing only p53, and yeast co-expressing p53 and MDM2. Results were estimated considering 100% growth, the number of CFU obtained with control yeast (empty vectors). The growth of yeast expressing MDM2 alone treated with compounds was approximately 100%; therefore it is represented by the control yeast. Data are mean \pm S.E.M. of five independent experiments; values obtained with yeast co-expressing p53 and MDM2 treated with the compound significantly different from DMSO only ($*P < 0.05$). **(B)** Effects of 10 μ M nutlin-3a and 10 μ M xanthonones **15** and **16** on the reversion of p53-induced S-phase cell cycle arrest by MDM2. Yeast cell cycle phases were quantified by flow cytometry; data are mean \pm S.E.M. of two independent experiments; values significantly different from DMSO only ($*P < 0.05$). **(C)** Effects of 1–100 μ M xanthonones **15** and **16** on the reduction of p53-induced growth inhibition by MDM2 in yeast co-expressing p53 and MDM2. Data are mean \pm S.E.M of four independent experiments; values significantly different from DMSO only ($*P < 0.05$). **(D)** Effects of 10 μ M nutlin-3a and 10 μ M xanthonones **15** and **16** on p53-dependent transcriptional activity by dual-luciferase yeast p53 transactivation assay, using the p53 RE PUMA fused to a luciferase gene. Results are plotted as relative to the transcriptional activity achieved by p53 alone (set to 100%). Data are mean \pm S.E.M of four independent experiments; values of yeast co-expressing p53 and MDM2 treated with the compound significantly different from DMSO only ($*P < 0.05$).

The effect of α -mangostin (**15**) and gambogic acid (**16**) on the negative regulation of p53-dependent transcriptional activity by MDM2 was also analysed in yeast using a previously reported dual-luciferase p53 transactivation assay (Andreotti et al., 2011). This assay was carried out in yeast cells co-expressing p53 and MDM2 that contained the p53 RE derived from the *BBC3/ PUMA* gene fused to a luciferase reporter. At 10 μ M, α -mangostin (**15**) and gambogic acid (**16**) scored in a similar manner to nutlin-3a, resulting in a significant increase of luciferase activity; that is, these xanthonones counteracted the inhibitory effect of MDM2 on p53-dependent transcriptional activity (Fig. 20D).

3.2.2. α -Mangostin and gambogic acid reactivate the p53 activity in human tumour cells

The molecular mechanism of action of α -mangostin (**15**) and gambogic acid (**16**) as inhibitors of the p53–MDM2 interaction was further ascertained in a human adenocarcinoma-derived MCF-7 tumour cell line with wt p53 and overexpression of MDM2. For this, a p53 reporter assay, in which the synthetic p53 RE pG13 is fused to the luciferase reporter gene, was performed. The results obtained showed that, like 10 μ M nutlin-3a, 10 μ M of α -mangostin (**15**) and gambogic acid (**16**) significantly increased the

luciferase activity in MCF-7 cells (Fig. 21). These results demonstrated that α -mangostin (**15**) and gambogic acid (**16**) increased the p53-dependent transcriptional activity, therefore inhibiting the negative effect of MDM2 on p53, in a human tumour cell line.

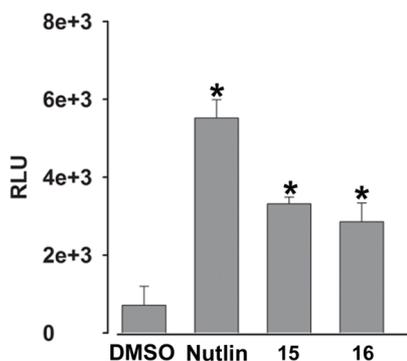


Figure 21. α -Mangostin (15**) and gambogic acid (**16**) increase the p53-dependent transcriptional activity in MCF-7 tumour cell line.** For the dual-luciferase gene reporter assay, the MCF-7 tumour cell was transfected with the pG13-luciferase reporter vector and was incubated in the presence of 10 μ M nutlin-3a and 10 μ M xanthenes 15 and 16 or DMSO only for 16 h. Nutlin-3a was used as positive control. RLU, relative light units. Data are mean \pm S.E.M of three independent experiments; values significantly different from DMSO only (* P < 0.05).

3.2.3. α -Mangostin and gambogic acid do not interfere with mutant p53 activity in yeast

In a previous study, it was shown that α -mangostin (**15**) was cytotoxic in human tumour cells harboring a mutant p53 form, such as in adenocarcinoma MDA-MB-231 tumour cells with the mutant p53 R280K (Shibata et al., 2011). Based on this, in the present work, the activity of α -mangostin (**15**) and gambogic acid (**16**) on mutant p53 R280K was also investigated using a yeast-based growth assay (Fig. 22). In contrast to wt p53, the expression of mutant p53 in yeast (Fig. 22A) did not interfere with the cell growth (similar to control yeast). Therefore, in this assay, activators of mutant p53 would reestablish the wt p53-dependent growth inhibition. The results obtained showed that both α -mangostin (**15**) and gambogic acid (**16**) had no impact on the growth of yeast expressing mutant p53 (Fig. 22B). These results therefore indicated that xanthenes **15** and **16** did not interfere with the mutant p53 activity.

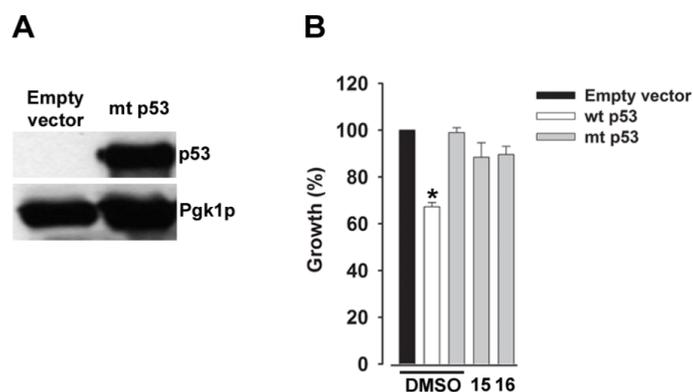


Figure 22. α -Mangostin (15) and gambogic acid (16) do not interfere with the activity of mutant p53. Yeast cells were incubated in the presence of 10 μ M xanthones **15** and **16** or DMSO only, for 31 h. **(A)** Expression of human mutant p53 (R280K; mt) in yeast was confirmed by Western blot analysis. Pgk1p was used as loading control. Immunoblots represent one of two independent experiments. **(B)** Effects of 10 μ M xanthones **15** and **16** on the growth of yeast expressing mutant p53. Results are plotted setting as 100% growth the number of CFU obtained with control yeast (empty vector). Data are mean \pm S.E.M of four independent experiments; values significantly different from control yeast (* $P < 0.05$).

3.2.4. Analysis of the predicted binding model of α -mangostin and gambogic acid to MDM2 supports that α -mangostin and gambogic acid binds to MDM2

With the absence of a direct effect of α -mangostin (**15**) and gambogic acid (**16**) on wt p53 activity (Fig. 22 A, B), the results obtained may indicate that as inhibitors of the p53-MDM2 interaction, α -mangostin (**15**) and gambogic acid (**16**) may act on MDM2. On the basis of the results obtained, a visual inspection of the potential for α -mangostin (**15**) and gambogic acid (**16**) to interact with MDM2 was performed by computational studies. Docking simulations in PyRx/AutoDock Vina allowed obtaining nine docked conformations for each ligand (Table A3). Nutlin-3a was used as positive control (Vassilev et al., 2004) and varacin, a known nonligand of MDM2 (Clement et al., 2008), was used as negative control. By computational docking against MDM2 (PDB code: 1YCR), the binding affinity values obtained for the best scoring conformations of both α -mangostin (**15**) and gambogic acid (**16**) were similar to those obtained for nutlin-3a (Table A3). Furthermore, analysis of the predicted binding model of α -mangostin (**15**) and gambogic acid (**16**) to MDM2 would indicate that these compounds adopted a pose within the p53-binding site, thus filling the space that provides for the interaction with α -helix motifs in the p53 amino terminal domain (Fig. 23). α -Mangostin (**15**) and gambogic acid (**16**) revealed, similarly to

nutlin-3a, affinity with *Gly58*, *Asp68*, *Val75*, and *Cys77*; the interacting residues included the hydrophobic region of the MDM2 binding site. Nonetheless, only gambogic acid (**16**) was predicted to establish hydrogen interactions with *Gln72* and *Phe55* residues of MDM2. These results are in accordance with previous findings (Warner et al., 2012), where nutlin-3a was predicted to contact nine MDM2 amino acids, but all without hydrogen interactions.

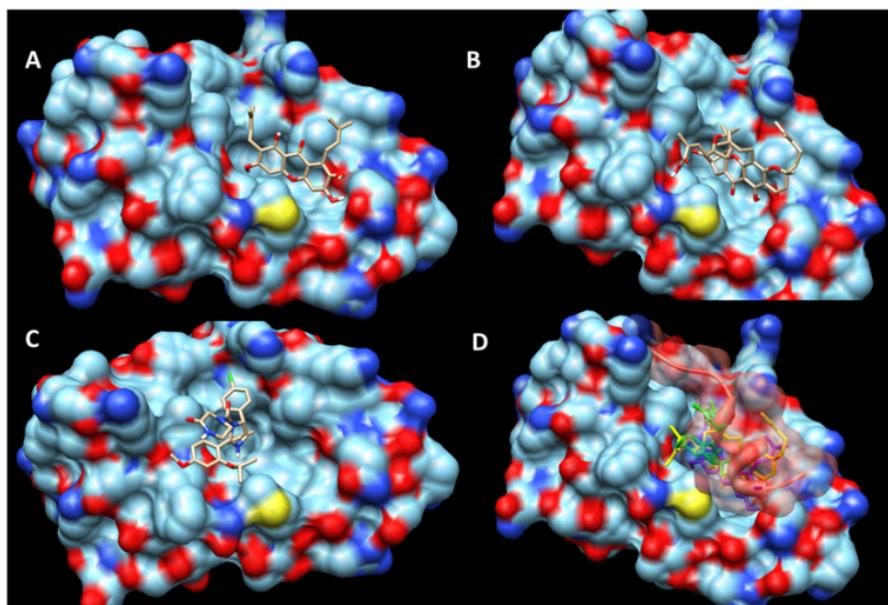


Figure 23. Predicted binding model using computational docking for α -mangostin (15**) and gambogic acid (**16**).** Best docking poses of (A) α -mangostin (**15**), (B) gambogic acid (**16**), and (C) nutlin-3a (positive control) on MDM2 (PDB code: 1YCR). (D) Superimposition of the best conformations of xanthenes **15** (purple), **16** (yellow), and nutlin-3a (green) on MDM2; the transparent surface represents the p53 helix.

3.2.5. Discussion

Using a simple, selective and reliable yeast growth-inhibitory assay developed to search for new inhibitors of the p53-MDM2 interaction, the xanthone scaffold was identified, for the first time, as a chemotype that blocks this interaction. Based on this, since α -mangostin and gambogic acid have both a xanthone scaffold, it was investigated if these two natural products are able to disrupt the p53-MDM2 interaction.

For instance, using the yeast growth-inhibitory screening assay followed by a yeast p53 transactivation assay (Andreotti et al., 2011), α -mangostin and gambogic acid were identified as potential inhibitors of the p53-MDM2 interaction. Furthermore, to ascertain whether α -mangostin and gambogic acid have effects on tumour cells consistent with an

inhibition of the p53-MDM2 interaction, these natural compounds were analysed in the human MCF-7 tumour cell line with wt p53. In fact, a correlation between the cytotoxic activity of these xanthenes and the activation of a p53-dependent pathway has been established in previous investigations (Gu et al., 2008; Rong et al., 2009; Aisha et al., 2012). For instance, for α -mangostin, it was shown that this compound increases the p53 transcriptional activity (Aisha et al., 2012) and induces apoptosis associated with an increase of the protein levels of Bax, a p53 target gene (Kaomongkolgit et al., 2008). Likewise, it was demonstrated that gambogic acid induces apoptosis and cell cycle arrest in human tumour cells harboring wt p53 (Gu et al., 2008). In this way, the results obtained in the present work confirmed the effectiveness of these two xanthenes in tumour cells with wt p53. Additionally, by computational docking studies, it was shown that, similarly to nutlin-3a, α -mangostin and gambogic acid bind to MDM2.

In spite of the effect of these two compounds on tumour cells with wt p53, it was demonstrated that α -mangostin induces apoptosis in human tumour cell lines with mutant forms of p53 (Shibata et al., 2011). Additionally, it was also shown that gambogic acid induces apoptosis and cell cycle arrest in human tumour cells without p53 (Rong et al., 2009). Based on this, the activity of α -mangostin and gambogic acid on a mutant form of p53 was analysed, indicating that the two xanthenes did not interfere with the mutant p53 activity.

Altogether, the results obtained in this investigation reveal that α -mangostin and gambogic acid are potential inhibitors of the p53-MDM2 interaction. Additionally, these results provide new clues and open new perspectives into the mode of action of these xanthenes in human tumour cells with wt p53.

Chapter 3.3.

Novel simplified yeast-based assays of regulators of the p53-MDMX interaction and p53 transcriptional activity

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With the reconstitution of the p53-MDM2 network in yeast, a new cell-based screening assay was developed, which led to the identification of new inhibitors of the p53-MDM2 interaction (Chapters 3.1. and 3.2.).

In this work, the human p53-MDMX regulatory pathway was also reconstituted in yeast. With this, an efficient yeast-based screening assay to search for inhibitors of the p53-MDMX interaction was developed for the first time. Beyond this, in the present work, further insights on p53 transcriptional activity in yeast are also provided.

3.3.1. Development of a yeast-based assay to search for inhibitors of the p53-MDMX interaction

In order to also study the regulatory effect of MDMX on p53 activity in yeast, the human MDMX was expressed in these cells either alone or combined with human wt p53 (Fig. 24A). Similarly to MDM2, although the single expression of MDMX in yeast did not interfere with the cell growth and cell cycle progression, its co-expression with p53 significantly reduced the p53-induced growth inhibition (Fig. 24B, DMSO) and S-phase cell cycle arrest (from $18.0 \pm 1.2\%$, obtained with cells expressing only p53, to $5.9 \pm 1.9\%$, $n = 2$, $P < 0.05$; Fig. 24C, DMSO). These results revealed the conservation in yeast of the negative effect of MDMX on p53 activity. Moreover, they indicated that yeast was a potential cell system to develop a simplified cell-based screening assay to search for inhibitors of the p53-MDMX interaction.

In order to analyse the efficacy of this assay to screen for inhibitors of the p53-MDMX interaction, the effect of the known inhibitor of the p53-MDMX interaction, SJ-172550 (Reed et al., 2010), was tested. In this assay, inhibitors of the p53-MDMX interaction would abolish or significantly reduce the negative effect of MDMX on p53 activity. As expected, 20 μM SJ-172550 significantly reduced the negative effect of MDMX on p53-induced growth inhibition (Fig. 24B) and S-phase cell cycle arrest (from $5.9 \pm 1.9\%$, obtained with DMSO only, to $15.8 \pm 1.5\%$, $n = 2$, $P < 0.05$; Fig. 24C), without interfering with the activity of p53 and MDMX when expressed alone (Fig. 24B, C). In fact, after 42 h treatment with 20 μM SJ-172550, approximately 83% of the p53-induced growth inhibition and S-phase cell cycle arrest were re-established. Higher concentrations of SJ-172550 did not improve the reversion of the MDMX effect on p53-induced growth inhibition (Fig. 24D). Besides, from 50 μM SJ-172550 a cytotoxic effect on control yeast (transformed with the empty vectors) was observed (Fig. 24E). Concentrations of SJ-172550 lower than 20 μM did not significantly reduce the negative effect of MDMX on p53-

induced growth inhibition (Fig. 24D). Additionally, SJ-172550 had no impact on the growth of yeast expressing p53 alone in the 0.1 - 20 μM concentration range (Fig. 24E).

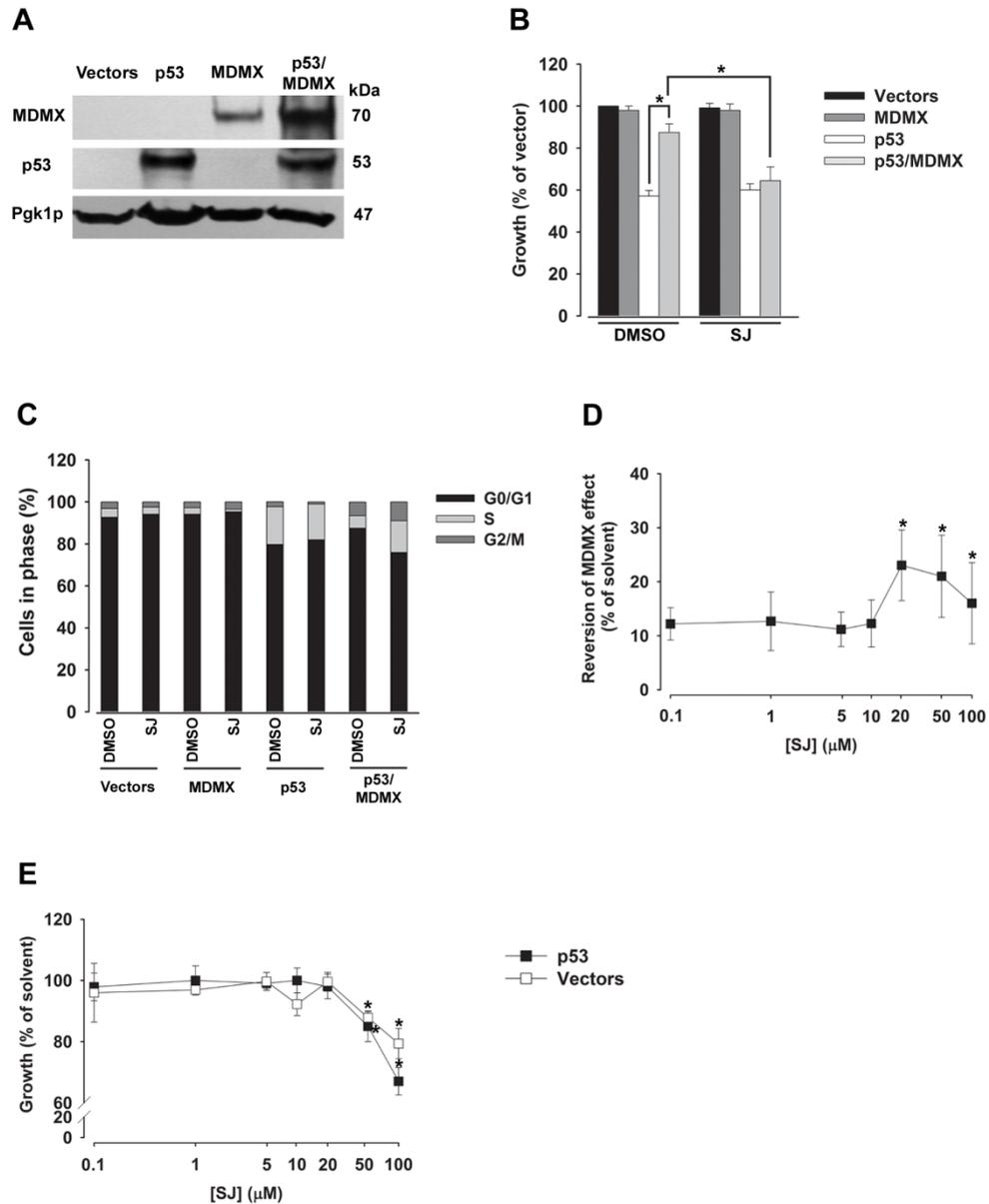


Figure 24. MDMX inhibits the impact of p53 on the growth of yeast, an effect abolished by SJ-172550. Yeast co-expressing human wt p53 and/or MDMX and control yeast (empty vectors) were incubated in selective induction medium for approximately 42 h. **(A)** Expression of human wt p53 and/or human MDMX was confirmed by Western blot analysis; Pgk1p was used as loading control; immunoblots represent one of two independent experiments. **(B)** Effect of 20 μM SJ-172550 on the growth of control yeast, yeast expressing only p53 or MDMX and yeast co-expressing p53 and MDMX; results were plotted setting as 100% growth the number of CFU obtained with control yeast incubated with DMSO only; data are mean \pm S.E.M. of five independent experiments; significantly different values are indicated (* $P < 0.05$). **(C)** Effect of 20 μM SJ-172550

on the yeast cell cycle progression of control yeast, yeast expressing only p53 or MDMX and yeast co-expressing p53 and MDMX; data are mean \pm S.E.M. of two independent experiments. **(D, E)** Concentration-response curves for the: **(D)** reversion of MDMX effect by SJ-172550 in yeast co-expressing p53; **(E)** effects of SJ-172550 on the growth of control yeast (transformed with the empty vectors) and yeast cells expressing p53 alone. Yeast cell growth was analysed by CFU counts after incubation in the presence of 0.1 - 100 μ M SJ-172550 or DMSO only; data are mean \pm S.E.M. of four independent experiments; values significantly different from DMSO only (* $P < 0.05$).

3.3.2. Expression of p53 in yeast increases actin protein and mRNA levels and induces actin depolarization

In the present work, it was detected that the growth inhibition caused by expression of human wt p53 in yeast (Fig. 24A, B) was also associated with a marked increase of actin protein levels, when compared to control yeast (transformed with the empty vector) (Fig. 25C).

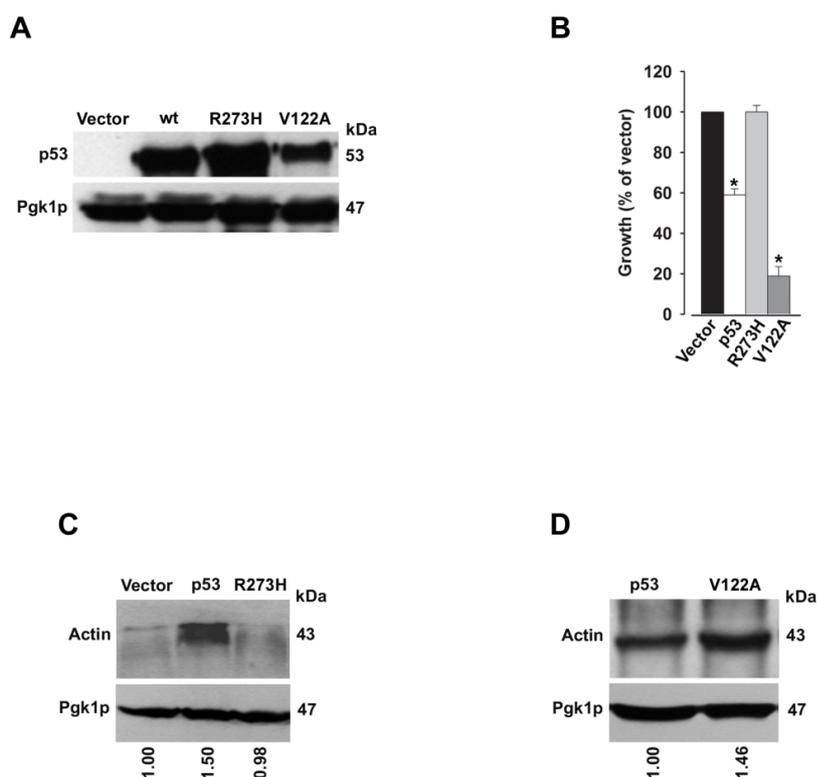


Figure 25. Contrary to R273H, wt p53 and V122A increase the actin protein levels. Yeast cells expressing human wt p53, mutant p53 R273H (R273H) or the toxic mutant p53 V122A (V122A) and control yeast (empty vector) were grown in selective induction medium for approximately 30 h. **(A)** Expression of human wt p53, R273H or V122A in yeast was confirmed by Western blot analysis. **(B)** Effect of wt p53, R273H and V122A on yeast cell growth was analysed by CFU

counts; results were plotted setting as 100% growth the number of CFU obtained with control yeast; data are mean \pm S.E.M. of five independent experiments; values significantly different from control yeast ($*P < 0.05$). (C, D) Effect of wt p53, R273H and V122A on actin protein levels was analysed by Western blot. Immunoblots represent one of two experiments; Pgk1p was used as loading control.

Instead, when the mutant p53 R273H (R273H), known for its reduced transcriptional activity in comparison to wt p53 both in mammalian cells (Maslon and Hupp, 2010) and in a yeast reporter construction (Schärer and Iggo, 1992), was expressed in yeast (Fig. 25A), no increase of actin protein levels was observed (Fig. 25C). Accordingly, R273H did not interfere with the yeast cell growth (Fig. 11B) and cell cycle progression (data not shown). Interestingly, when the toxic mutant p53 V122A (V122A), reported to have higher cytotoxic and transcriptional activity than wt p53 (Inga and Resnick, 2001), was expressed in yeast (Fig. 25A), its higher inhibitory effect on yeast cell growth (Fig. 25B) was accompanied by a higher increase of actin protein levels (Fig. 25D), when compared to wt p53.

In this work, the inhibitory effect of PFT- α on the p53-dependent transcriptional activity was further confirmed using the reported luciferase yeast p53 transactivation assay (Andreotti et al., 2011). This assay was performed in yeast cells expressing wt p53, and used the p53 RE derived from the human *BBC3/PUMA* gene fused to a luciferase reporter. As expected, a dose-dependent inhibitory effect of PFT- α on p53-dependent transcriptional activity was obtained (Fig. 26A). Additionally, when yeast cells expressing wt p53 were treated with 20 μ M PFT- α , a prominent reduction of p53-induced growth inhibition of $41.7 \pm 4.3\%$ ($n = 4$) was observed as previously reported (Coutinho et al., 2011), which was accompanied by a significant reduction of the actin protein levels when compared to cells treated with DMSO only (Fig. 26B).

Since it was shown that MDM2 and MDMX also inhibited the toxic effect of p53 in yeast, the impact of these two negative regulators on p53-induced actin protein levels was also analysed. Although the single expression of human MDM2 and MDMX in yeast did not interfere with the actin protein levels (data not shown), when co-expressed with wt p53, both MDM2 (Fig. 26C) and MDMX (Fig. 26D) led to a visible reduction of actin protein levels. Interestingly, the treatment of yeast cells co-expressing p53 and MDM2 with 10 μ M of the known inhibitor of the p53-MDM2 interaction, nutlin-3a, reverted this inhibitory effect of MDM2, re-establishing the p53-induced actin protein levels (Fig. 26C). Similarly to nutlin-3a with MDM2 co-expressing cells, 20 μ M SJ-172550 re-established the p53-induced actin protein levels in yeast cells co-expressing p53 and MDMX (Fig. 26D).

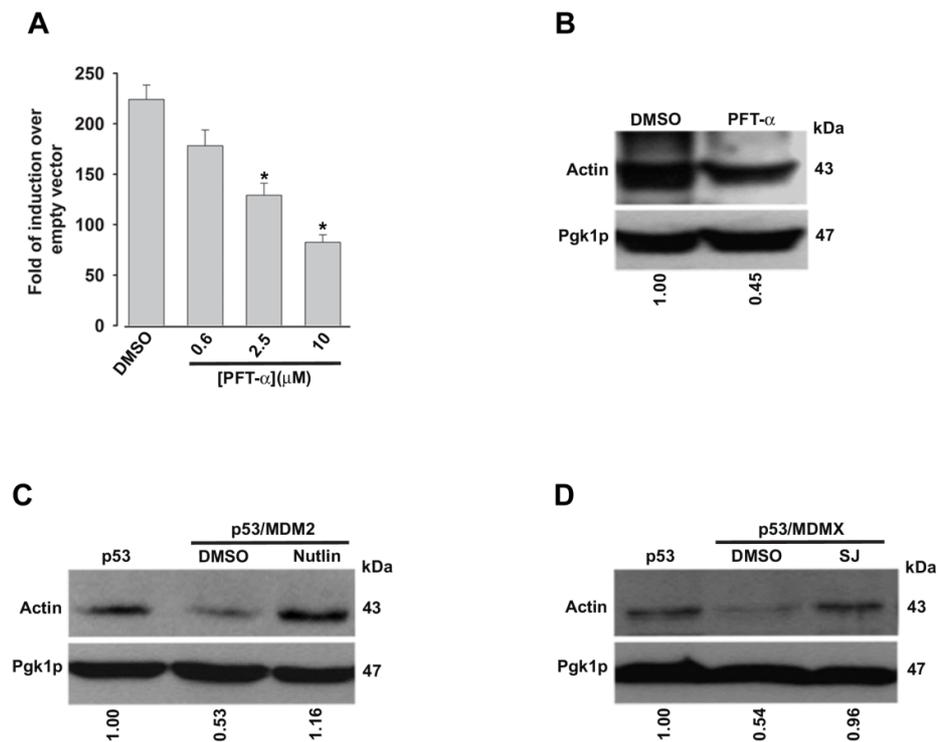


Figure 26. Reduction of p53-induced actin protein levels by chemical (PFT- α) and natural (MDM2 and MDMX) inhibitors of p53 activity. (A) Effect of PFT- α on the p53-dependent transcriptional activity; yeast cells expressing wt p53 were treated with 0.6, 2.5 and 10 μ M PFT- α , for 16 h; yeast p53 transactivation assay was carried out using the p53 RE derived from the human *BBC3/PUMA* gene fused to the *Firefly* luciferase gene; results are plotted as fold of reporter activation compared to control yeast (empty vector); data are mean \pm S.E.M. of four independent experiments; values significantly different from DMSO only (* P < 0.05). (B) Effect of PFT- α on p53-induced actin protein levels was analysed by Western blot; yeast cells expressing wt p53 were grown in selective induction medium for approximately 30 h in the presence of 20 μ M PFT- α or DMSO only. (C) Effect of MDM2 on p53-induced actin protein levels was analysed by Western blot; yeast cells co-expressing human wt p53 and/or MDM2 and control yeast (empty vectors) were grown in selective induction medium for approximately 42 h in the presence of 10 μ M nutlin-3a or DMSO only. (D) Effect of MDMX on p53-induced actin protein levels was analysed by Western blot; yeast cells co-expressing human wt p53 and/or MDMX and control yeast (empty vectors) were grown in selective induction medium for approximately 42 h in the presence 20 μ M SJ-172550 or DMSO only. Immunoblots represent one of two experiments; Pgk1p was used as loading control.

Altogether, these results suggested that *ACT1* was an endogenous p53 target gene in yeast. To further support these results, the actin mRNA levels were determined by Q-PCR in yeast cells expressing wt p53 and control yeast (vector). In accordance with the increase of actin protein levels obtained by Western blot analysis, a 1.5-fold increase of

actin mRNA levels was obtained with yeast cells expressing wt p53, when compared to control yeast (Fig. 27A). To further investigate the possibility of a direct p53-dependent transcriptional modulation of the *ACT1* promoter, a pattern search analysis was conducted revealing a putative p53 RE upstream the *ACT1* open reading frame (<http://www.genomatix.de>). The sequence comprises a half site RE and a mismatched full-site RE separated by 38 nucleotides (Fig. 27B). This fragment was cloned and placed upstream of a minimal promoter regulating the expression of the *Firefly* luciferase gene using an available yeast strain (Andreotti et al., 2011). A mutated version of the sequence was also constructed (Fig. 27B). The strains were then transformed with an empty vector or with vectors expressing either wt p53 or the mutant R273H under the inducible *GAL1* promoter, and the activation of the reporter was measured in galactose media. The putative p53 RE from *ACT1* exhibited responsiveness to wt p53 (although weak), but not to mutant R273H (Fig. 27C). A mutation in the conserved CATG motif within the RE abolished the responsiveness to p53. Overall, the results support a direct effect of p53 on *ACT1* gene expression although the low-level of responsiveness does not allow us to rule out that the observed actin protein levels changes could be in part dependent from indirect effects of the overexpression of functional p53 in yeast.

To explore the possibility that wt p53 could impact on actin mRNA translation efficiency, polysome-associated RNA was separated from cells transformed with an empty vector as well as a p53-expression vector (Arava, 2003). mRNA was recovered and Q-PCR revealed a p53-dependent increase of actin mRNA in actively translating polysomes (Fig. 27D).

The effect of p53 on the yeast actin cytoskeleton organization was also checked in this work. Yeast cells expressing wt p53 were co-transformed with a yeast integrative vector encoding the GFP fused to the actin binding protein 140 (ABP140), as described (Sagot et al., 2006). The actin organization was thereafter visualized by fluorescence microscopy. The results obtained showed that expression of p53 in yeast led to a marked actin cytoskeleton depolarization after 16 h incubation in induction selective medium. In fact, contrary to the diffuse GFP cellular background corresponding to the staining of actin cables observed in control yeast (vector), highly depolarized actin patches confined to regions near the plasma membrane could be visualized in a high number of yeast cells expressing wt p53 (Fig. 27E, F; 30 h incubation).

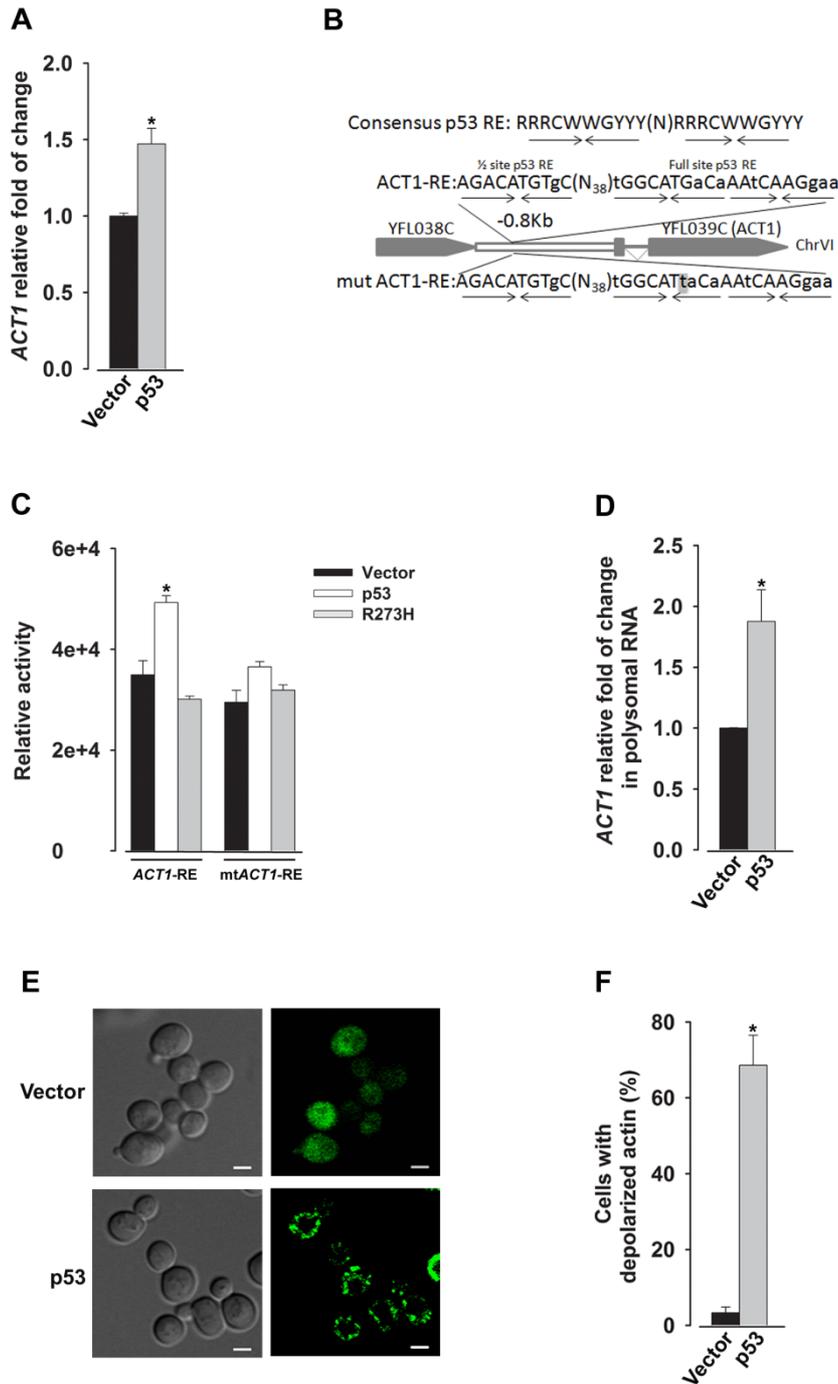


Figure 27. Expression of p53 in yeast increases actin mRNA levels in total RNA and polysome-associated RNAs, stimulate transcription in *ACT1*-promoter reporter assays, and induces actin depolarization. Yeast cells expressing wt p53 and control yeast (empty vector) were incubated in induction selective medium for approximately 30 h. **(A)** Effect of p53 on actin mRNA levels was determined by Q-PCR; data are mean \pm S.E.M. of three independent experiments. **(B)** Schema of the *ACT1* region on Chr VI of *S. cerevisiae*. The position and the sequence of the putative p53 RE are indicated. The consensus p53 RE is shown; mismatches from the p53 RE consensus are in lowercase. In mutant *ACT1*-RE the mutation affecting the conserved G of the CATG motif of the full site RE is highlighted in gray. **(C)** Effect of wt p53 and R273H on the

transcription of wt and mutant *ACT1*-derived p53 RE. Data are mean \pm S.E.M. of four independent experiments. The activity of the *Firefly* luciferase reporter gene is normalized using the optical density of the cell cultures (Andreotti et al., 2011). Values significantly different from empty vector ($*P < 0.05$). (D) Effect of p53 on actin mRNA levels associated with actively translating polysomes. Polysome-associated mRNAs were recovered upon sucrose-gradient transformation of yeast cells cultured in galactose medium to induce the expression of p53. Data are mean \pm S.E.M. of three technical replicates from one independent experiment; values significantly different from empty vector ($*P < 0.05$). (E) Effect of p53 on yeast actin organization was analysed by fluorescence microscopy using yeast expressing wt p53 or control yeast co-transformed with the yeast integrative ABP140-3xGFP plasmid. Bars, 2 μ m. (F) Quantification of actin depolarization was estimated by counting at least 300 cells per sample; data are mean \pm S.E.M. of two independent experiments; value significantly different from control yeast ($*P < 0.05$).

3.3.3. Discussion

With the reconstitution of human p53-MDMX regulatory pathway in yeast, a simplified and cost-effective yeast-based growth-inhibition assay to search for inhibitors of the p53-MDMX interaction was developed for the first time. This assay can be easily adapted to the HTS of large chemical libraries based on simple measurements of the yeast cell growth by optical density. In fact, several advantages (pointed out in Chapter 1, Table 1) may justify the success achieved by engineered yeast cells in the development of fast, selective and reliable HTS assays. Additionally, the possibility for a more direct analysis of simplified networks of human proteins without orthologues in this microorganism (particularly p53 and MDM2/MDMX) may eliminate the effects of redundant processes on the measured output [reviewed in (Pereira et al., 2012a, b)]. The developed yeast assay can be greatly useful in the first-line selection of potential active compounds to be tested in more complex cell models. This will certainly reduce the costs and may expedite the discovery of new inhibitors of the p53-MDMX interaction.

A previous work from Comer and colleagues performed in human cells (Comer et al., 1998) showed that the p53-induced growth inhibition and cell cycle arrest was associated with drastic changes in the actin cytoskeleton organization and with an increase of smooth muscle α -actin mRNA levels. More recently, it was also reported that nutlin-3 was a potent inducer of α -actin expression in human cells (Secchiero et al., 2012). These works showed that actin, with a crucial role in cell cycle progression [reviewed in (Heng and Koh, 2010)], was a direct p53 transcriptional target. Interestingly, similar results were obtained in yeast. In fact, in the present work, it was shown that expression of p53 in yeast was associated with an increase of actin mRNA and protein levels, as well as with

actin cytoskeleton depolarization. It was also observed that p53-induced actin protein levels were modulated by natural (MDM2 and MDMX) and chemical (PFT- α , nutlin-3a and SJ-172550) regulators of p53 activity. Together, these results revealed, for the first time, that *ACT1* was a putative p53 target gene in yeast.

Yeast p53 transactivation assays have been extensively used by several authors to study the impact of mutations, cofactors and small molecules on p53 transcriptional activity. However, these assays have been carried out using artificial yeast reporter constructs with response elements derived from human genes regulated by p53, such as *PUMA* and *BAX* [reviewed in (Pereira et al., 2012a, b)]. With the identification of *ACT1* as an endogenous p53 target gene in yeast, a simpler yeast p53 transactivation assay may be used for the analysis of p53 transcriptional activity.

Altogether, with this work, relevant insights on p53 regulatory pathway in yeast are revealed, which may open the way to new simplified and effective yeast-based assays for functional and pharmacological studies of the p53-MDMX network and p53 transcriptional activity.

Chapter 3.4.

Studying p53 family proteins in yeast: induction of autophagic cell death and modulation by interactors and small molecules

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In the present work, the yeast *S. cerevisiae* was used as a simplified model system to study individual p53 family members. Several similarities among the activities of the different p53 family proteins expressed in yeast were found in this work. Despite this, member- or even isoform-specific effects were pointed out by our cell system, particularly to the autophagic pathway and to the inhibitory effects of MDM2 and MDMX. Most importantly, in this chapter, several yeast assays were developed, which may contribute to the elucidation of the biology and pharmacology of p53 family proteins, particularly to study the impact of cofactors and small molecules on their activities.

3.4.1. TAp63, Δ Np63 and TAp73 induce yeast growth inhibition and cell cycle arrest associated with an increase of actin expression levels and depolarization

In order to evaluate the impact of TAp63, Δ Np63 and TAp73 on yeast growth, yeast cells were transformed with the appropriate expression vectors. Human wt p53 and mutant p53 R273H (mt p53; a loss of function p53 mutation hotspot) were used as positive and negative controls, respectively.

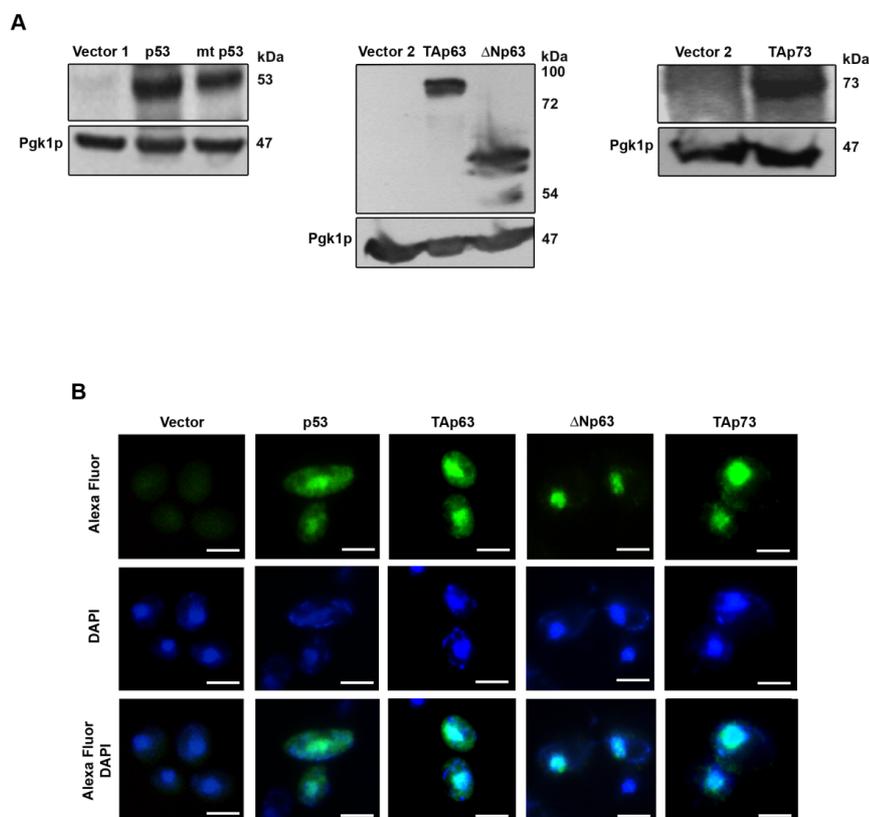


Figure 28. Nuclear localization of p53 family members expressed in yeast. Yeast cells individually expressing human p53, mt p53, TAp63, Δ Np63 or TAp73 and control yeast (vector) were grown in induction selective medium until the corresponding control yeast reached 0.4 OD₆₀₀.

(A) Expression of human p53, TAp63, Δ Np63 and TAp73 in yeast was confirmed by Western blot analysis. Pgk1p was used as loading control. Immunoblots are representative of two independent experiments. (B) Subcellular localization of p53, TAp63, Δ Np63 and TAp73 in yeast was assessed by immunofluorescence microscopy using Alexa Fluor 488 secondary antibody; nuclear localization was visualized using DAPI. Bars, 10 μ m.

Firstly, the expression of each human protein (p53, mt p53, TAp63, Δ Np63 and TAp73) in yeast was confirmed by Western blot analysis using appropriate antibodies (Fig. 28A). Immunofluorescence analyses confirmed that, as previously described for p53 (Coutinho et al., 2011), also human TAp63, Δ Np63 and TAp73 were predominantly localized in the nucleus of yeast cells (Fig. 28B).

Then, the impact of the p53 family members on yeast growth was evaluated. While the expression of p53 caused, with respect to the control (vector 1), a marked growth inhibition (Figs. 29A, B) associated with S-phase cell cycle arrest (Fig. 29C), confirming previous results (Coutinho et al., 2009), mt p53 did not interfere with cell growth and cell cycle progression (Fig. 29A-C). Similarly to p53, TAp63, Δ Np63 and TAp73 induced, with respect to the control (vector 2), yeast growth inhibition (Fig. 29A, B) associated with cell cycle arrest in S-phase (Fig. 29C). Interestingly, among the p53 family members tested, TAp63 exhibited the highest growth inhibitory effect in yeast (Fig. 29A, B).

In the previous work (Chapter 3.3.), it was shown that p53-induced yeast growth inhibition and cell cycle arrest were accompanied by an increase of actin depolarization and actin expression levels. Using Western blot, we determined that also TAp63, Δ Np63 and TAp73 increased the yeast actin expression levels, when compared to control yeast (vector 2) (Fig. 29D). In order to determine the impact of TAp63, Δ Np63 and TAp73 on the yeast cytoskeleton organization, yeast cells expressing each of the p53 family members were co-transformed with the yeast integrative vector encoding the green fluorescent protein (GFP) fused to the actin binding protein 140 (ABP140), and the actin organization was evaluated by fluorescence microscopy. As previously observed with p53 (Chapter 3.3.), after 24 h incubation, highly depolarized actin patches near the plasma membrane could be visualized in yeast cells expressing TAp63, Δ Np63 or TAp73, in contrast to the diffuse GFP background corresponding to the staining of actin cables observed in yeast transformed with control vector (Fig. 29E, left panel). However, from the quantitative point of view, it is noteworthy that the effect obtained with TAp63, Δ Np63 and TAp73 was about one half of that obtained with p53 in terms of percentage of cells with actin depolarization (Fig. 29E).

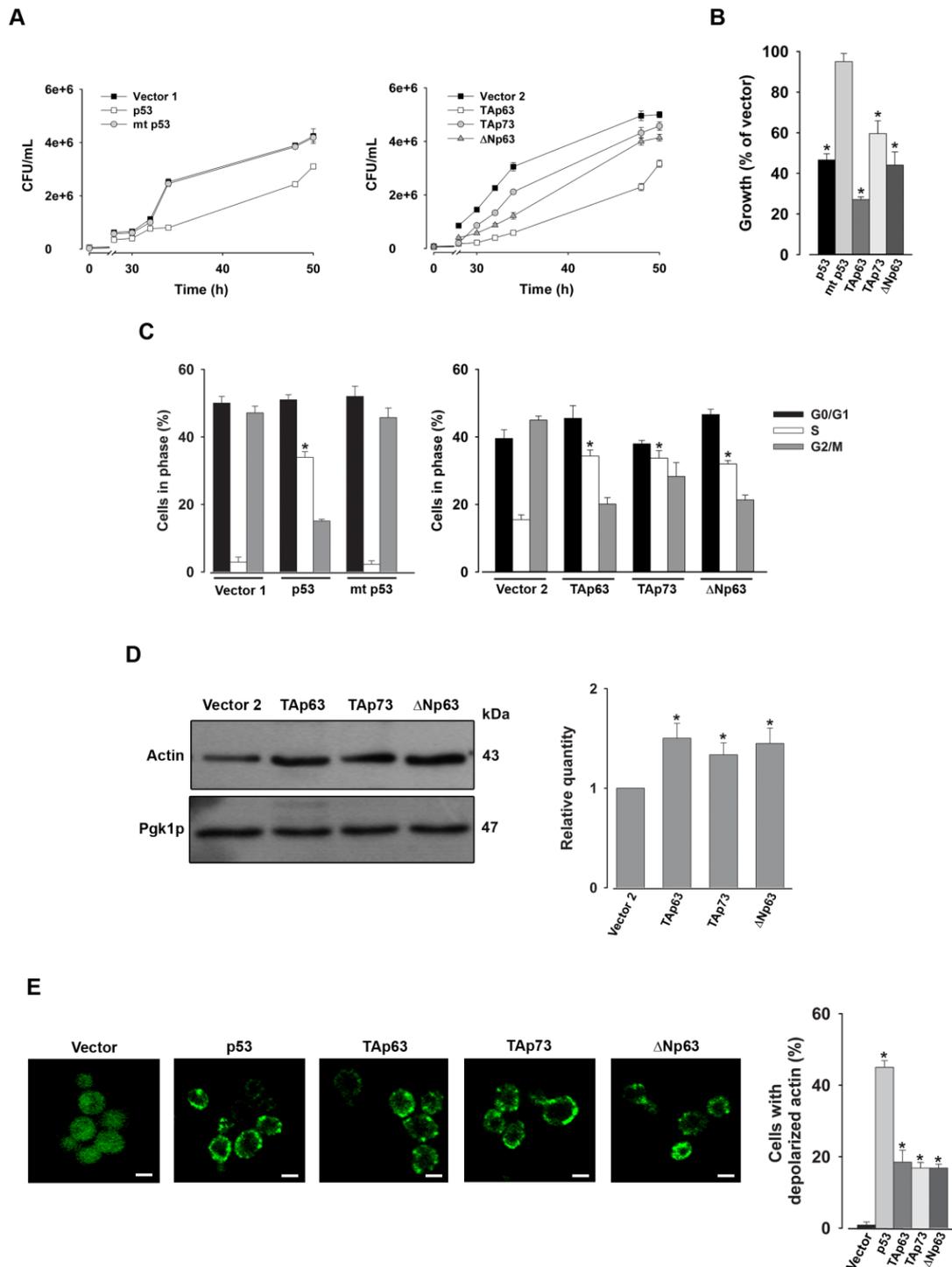


Figure 29. Expression of p53 family members in yeast induces growth inhibition associated with cell cycle arrest, an increase of actin expression levels and depolarization. Yeast cells individually expressing human p53, mt p53, TAp63, Δ Np63 or TAp73 and control yeast (vector) were grown in induction selective medium for up to 50 h for growth curves (**A**) or until the corresponding control yeast reached 0.4 OD₆₀₀, in all other experiments (**B – E**). (**A**) Growth curves were obtained by CFU counts; presented are the mean of two independent experiments with six replicates. Effects of p53, TAp63, Δ Np63 and TAp73 on yeast cell growth were significantly different from control yeast ($P < 0.0001$; two-way ANOVA). (**B**) Growth inhibition induced by p53,

mt p53, TAp63, Δ Np63 and TAp73. Results are plotted setting as 100% the number of CFU obtained with control yeast. Data are mean \pm S.E.M. of six independent experiments. (C) Effect of p53, mt p53, TAp63, Δ Np63 and TAp73 on yeast cell cycle progression. Data are mean \pm S.E.M. of two independent experiments; values significantly higher than control yeast ($*P < 0.05$). (D) Effect of TAp63, Δ Np63 and TAp73 on actin protein expression levels was analysed by Western blot. Pgk1p was used as loading control. Immunoblots represent one of two independent experiments. Values of band intensities were normalized to the vector, and are presented as mean \pm S.E.M. of two independent experiments; values significantly different from control yeast ($*P < 0.05$). (E) Effect of p53, TAp63, TAp73 and Δ Np63 on yeast actin organization was analysed by fluorescence microscopy using yeast cells, expressing a p53 family protein and control yeast, co-transformed with the yeast integrative ABP140-3xGFP plasmid (left panel; bars, 2 μ m). Quantification of actin depolarization was estimated by counting at least 300 cells per sample; yeast cells expressing p53 were used as positive control. Results presented for control yeast (vector) correspond to the mean values obtained with vectors 1 and 2. Data are mean \pm S.E.M. of two independent experiments. In (B) and (E), values significantly different from control yeast ($*P < 0.05$).

Taken together, the results showed that induction of yeast growth inhibition and cell cycle arrest by TAp63, Δ Np63 and TAp73 were associated with an increase of actin expression levels and with its depolarization.

3.4.2. Induction of an autophagic cell death by p53 family proteins in yeast

In order to deepen inside the p53 family growth inhibitory effect, clonogenic assays were used. The results showed that the p53-, TAp63-, Δ Np63- and TAp73-induced yeast growth inhibitory effects were also associated with cell death, which showed to be more marked for p53 and TAp63 (Fig. 30A). On the contrary, mt p53 did not affect the yeast viability (Fig. 30A). Additionally, when compared to control yeast (vector), the expression of p53, TAp63, Δ Np63 or TAp73, but not of mt p53, was associated with a significant stimulation of ROS generation (Fig. 30B). In spite of this, the expression of p53 family members in yeast did not increase, with respect to the control yeast (vector), neither apoptosis, evaluated by the percentage of cells with DNA fragmentation (TUNEL-positive cells) and with externalization of phosphatidylserine (Annexin V-positive cells), nor necrosis, evaluated by the percentage of cells with loss of plasma membrane integrity (PI-positive cells) (Fig. 30C). These results indicated that p53-, TAp63-, Δ Np63- and TAp73-induced yeast cell death was neither associated to apoptosis nor necrosis. To further investigate whether p53 family members might influence apoptosis not *per se* but after treatment with H₂O₂ or acetic acid, yeast cells individually expressing p53, mt p53, TAp63,

Δ Np63 or TAp73 and control yeast were treated with these known yeast apoptotic inducers (Fig. 30D). Confirming previous results obtained with H₂O₂-treated yeast cells expressing p53 (Coutinho et al., 2011), upon treatment with H₂O₂ or acetic acid, the expression of p53, mt p53, TAp63, Δ Np63 or TAp73 did not significantly increase the percentage of cell death induced by H₂O₂ or acetic acid, when compared to control yeast.

A possible involvement of autophagy in p53 family members-induced cell death was thereafter investigated. To this end, the Atg8p protein levels were checked in yeast expressing p53, mt p53, TAp63, Δ Np63 or TAp73 (Fig. 30E). In fact, this protein is essential for autophagosome formation and its increase, in most of the cellular contexts including in yeast, is an autophagic marker (Kirisako et al., 1999). A two-fold increase of Atg8p expression levels (protein band at 14 kDa) was observed in yeast cells expressing p53, TAp63, TAp73 or Δ Np63, but not in yeast cells expressing mt p53 (Fig. 30E). To further confirm the possible involvement of an autophagic process in the p53 family members-induced cell death, W303 yeast strains deficient in *ATG1* or *ATG5*, genes coding for proteins with a role in general autophagy, were used as recipient for the expression of p53, mt p53, TAp63, TAp73 or Δ Np63. Atg1p and Atg5p are proteins of the yeast autophagic pathway required for the autophagosome formation, and their deletion leads to a defective macroautophagy (Matsuura et al., 1997; George et al., 2000). Cell death assays with p53, mt p53, TAp63, TAp73 and Δ Np63 proteins were performed in a W303 background using three different strains (wt, *atg1* Δ ; *atg5* Δ) (Fig. 30F). The pattern of cell death obtained with wt W303 expressing p53, mt p53, TAp63, TAp73 or Δ Np63 proteins (Fig. 30F) was close to that obtained with the CG379 strain (Fig. 30A). Interestingly, the cell death observed with *atg1* Δ and *atg5* Δ W303 derivative strains expressing p53, TAp63, TAp73 or Δ Np63 was significantly reduced with respect to that observed with wt W303 (Fig. 30F). Particularly, an almost complete abolishment of Δ Np63-induced cell death was achieved with autophagy deficient strains with respect to wt. Once again, mt p53 did not affect the viability of any of the studied strains (Fig. 30F). Furthermore, when yeast cells expressing a p53 family member were treated with rapamycin, a well-known inducer of autophagy (Noda and Ohsumi, 1998), all the p53 family members, with the exception of mt p53, stimulated the rapamycin-induced cell death, being the effect more pronounced for p53 (Fig. 30G).

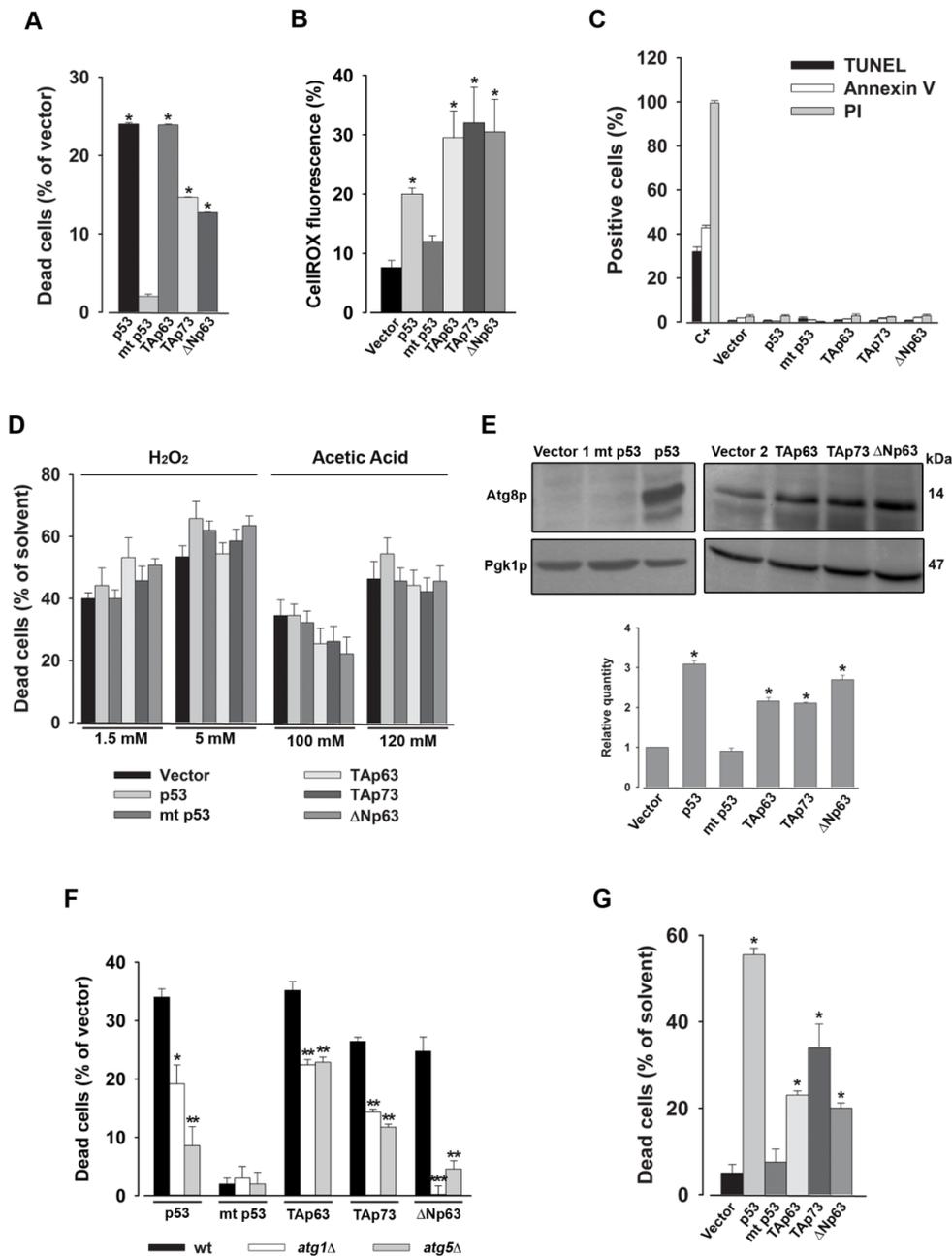


Figure 30. p53 family members induce ROS generation and an autophagic cell death in yeast. Yeast cells individually expressing human p53, mt p53, TAp63, Δ Np63 and TAp73 and control yeast (vector) were grown in induction selective medium until 0.4 OD₆₀₀ achieved by all the transformants in (D) and (G); achieved by the corresponding control yeast in all other experiments. (A) Effect of human p53 family members on cell death of wt CG379 strain. Percentage of dead cells was determined using the clonogenic assay; results are plotted setting as 100% survival (0% death) the number of CFU obtained with control yeast. Data are mean \pm S.E.M. of four independent experiments. (B) Quantification of ROS generation (CellROX Green) was performed by flow cytometry and is expressed as percentage of total cells. Data are mean \pm SEM of three independent experiments. (C) Percentage of apoptotic cells, with DNA fragmentation (TUNEL-

positive) and externalization of phosphatidylserine (Annexin V-positive), and necrotic cells (PI-positive) obtained with yeast expressing a p53 family member and control yeast. Cells treated with 1.5 mM of H₂O₂, 120 mM of acetic acid and boiled cells were used as positive controls for TUNEL, Annexin V and PI assays, respectively. Data are mean ± S.E.M. of four independent experiments. (D) Effect of human p53 family members on H₂O₂- and acetic acid-induced apoptotic cell death. Percentage of dead cells was determined after 1 h treatment with 1.5 and 5 mM H₂O₂ or 100 and 120 mM acetic acid; results are plotted setting as 100% survival (0% death) the number of CFU obtained with untreated cells. Data are mean ± S.E.M. of four independent experiments; values were not significantly different from control yeast ($P > 0.05$, unpaired Student's *t*-test). (E) Western blot analysis of Atg8p protein levels in yeast cells individually expressing a human p53 family member. Pgk1p was used as loading control. Immunoblots represent one of two independent experiments. Values of band intensities were normalized to the vector, and are presented as mean ± S.E.M. of two independent experiments; values significantly different from control yeast ($*P < 0.05$; unpaired Student's *t*-test). (F) Effect of human p53 family members on cell death of wt, *atg1Δ* and *atg5Δ* W303 strains. Percentage of dead cells was determined using the clonogenic assay; results are plotted setting as 100% survival (0% death) the number of CFU obtained with control yeast. Data are mean ± S.E.M. of four independent experiments. (G) Effect of human p53 family members on rapamycin-induced autophagic cell death. Percentage of dead cells was determined after 4 h treatment with 0.2 μg/mL of rapamycin; results are plotted setting as 100% survival (0% death) the number of CFU obtained with cells treated with the solvent (DMSO) only. Data are mean ± S.E.M. of four independent experiments. In **B**, **C**, **F** and **G**, results presented for control yeast (vector) correspond to the mean values obtained with vectors 1 and 2. Values significantly different from control yeast (**A**, **B**, **F** and **G**) or from wt strain (**E**) ($*P < 0.05$).

Altogether, these results show that p53 family members-induced growth inhibition was also associated with cell death, which in our experimental conditions was found to be predominantly autophagic.

3.4.3. MDM2 and MDMX inhibit the activity of TAp63, ΔNp63 and TAp73 in yeast

We previously demonstrated that expression of MDM2 (Chapter 3.1.) and MDMX (Chapter 3.3.) inhibit the p53 activity in yeast. Along this line, we wanted to determine whether TAp63, ΔNp63 and TAp73 activities were also inhibited by human MDM2 and MDMX in yeast. For this purpose, TAp63, ΔNp63 and TAp73 were singly expressed in yeast or concomitantly with human MDM2 and MDMX, as confirmed by Western blot (Fig. 31A, B). Although MDM2 and MDMX did not interfere *per se* with the cell growth and cell

cycle progression, similarly to that obtained with p53, these proteins significantly reduced the TAp63-, TAp73- and Δ Np63-induced growth inhibition (Fig. 31C) and S-phase cell cycle arrest (Fig. 31D).

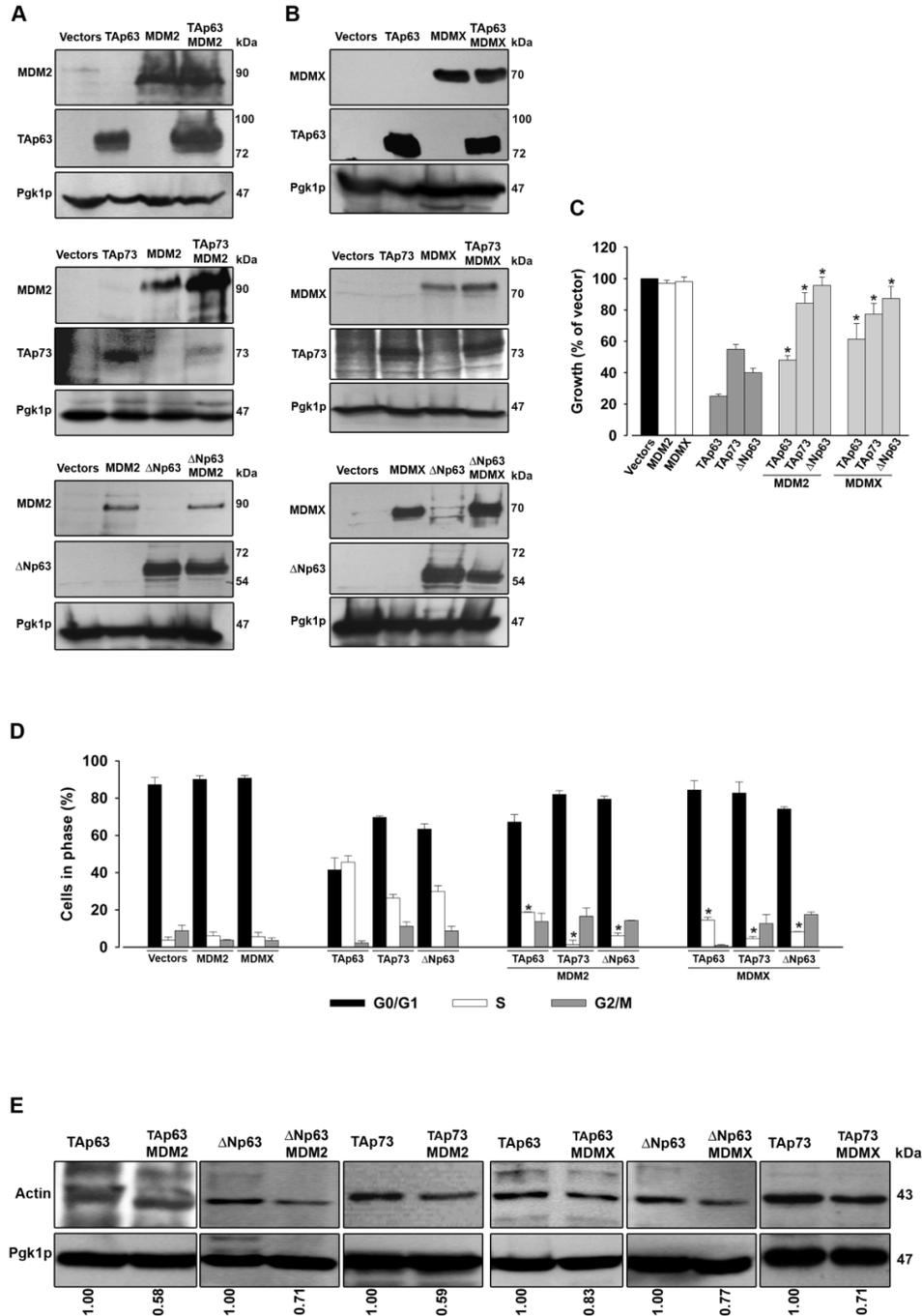


Figure 31. MDM2 and MDMX reduce the TAp63-, Δ Np63- and TAp73-induced growth inhibition, cell cycle arrest, and increase of actin expression in yeast. Yeast cells co-expressing human TAp63, Δ Np63 or TAp73 and/or human MDM2/MDMX, and control yeast (vectors) were grown in induction selective medium until the control yeast reached 0.4 OD₆₀₀. (A, B) Expression of TAp63, Δ Np63 or TAp73 alone and combined with MDM2 (A) or MDMX (B) in yeast was confirmed by Western blot analysis. Pgk1p was used as loading control. Immunoblots

represent one of two independent experiments. (C) Effect of MDM2 and MDMX on TAp63-, Δ Np63- and TAp73-induced growth inhibition was assessed by CFU counts. Results are plotted setting as 100% growth the number of CFU obtained with control yeast. Data are mean \pm S.E.M. of four independent experiments; values obtained with co-expression systems significantly different from the respective p53 family member expressed alone ($*P < 0.05$). (D) Effect of MDM2 and MDMX on TAp63-, Δ Np63- and TAp73-induced cell cycle arrest. Data are mean \pm S.E.M. of two independent experiments; values obtained with co-expression systems significantly lower than the respective p53 family member expressed alone ($*P < 0.05$). (E) Effect of MDM2 and MDMX on TAp63-, Δ Np63- and TAp73-induced actin protein levels were analysed by Western blot. Pgk1p was used as loading control. Immunoblots represent one of two independent experiments.

In spite of the qualitative effect of MDM2 and MDMX was conserved in yeast for all the p53 family members studied, the effect was much weaker on TAp63 (especially for MDM2) and stronger on Δ Np63 (Fig. 31C). Interestingly, the profiles of inhibition by MDM2 and MDMX were similar towards the same p53 family member (Fig. 31C, D). Additionally, although MDM2/MDMX did not interfere *per se* with the expression of actin (data not shown), it was shown that they were also able to inhibit the increase of actin expression levels caused by p53 in yeast. Similarly, when co-expressed with TAp63, TAp73 or Δ Np63, MDM2 and MDMX also inhibited the increase of actin expression levels caused by these p53 family proteins (Fig. 31E).

3.4.4. Nutlin-3a and SJ-172550 inhibit the negative effect of MDM2 and MDMX, respectively, on TAp73

The effect of nutlin-3a (inhibitor of the p53-MDM2 interaction) and of SJ-172550 (inhibitor of the p53-MDMX interaction), on the inhibitory effect of MDM2 and MDMX, respectively, over the activity of TAp63, Δ Np63 and TAp73 was thereafter analysed using co-expression in yeast. For the concentration range tested of 0.1-50 μ M nutlin-3a and 0.1-100 μ M SJ-172550, none of these compounds interfered with the inhibitory effect of MDM2 and MDMX, on TAp63- and Δ Np63-induced growth inhibition (Fig. 32A). On the contrary, as obtained with p53 (Chapters 3.1. and 3.3.; Fig. 32A), 10 μ M nutlin-3a and 20 μ M SJ-172550 significantly reduced the negative effect of MDM2 and MDMX, respectively, on TAp73-induced growth inhibition (Fig. 32A) and S-phase cell cycle arrest

(Fig. 32B), without interfering with the growth/cell cycle progression of yeast cells expressing TAp73 or MDM2/MDMX alone and of control yeast.

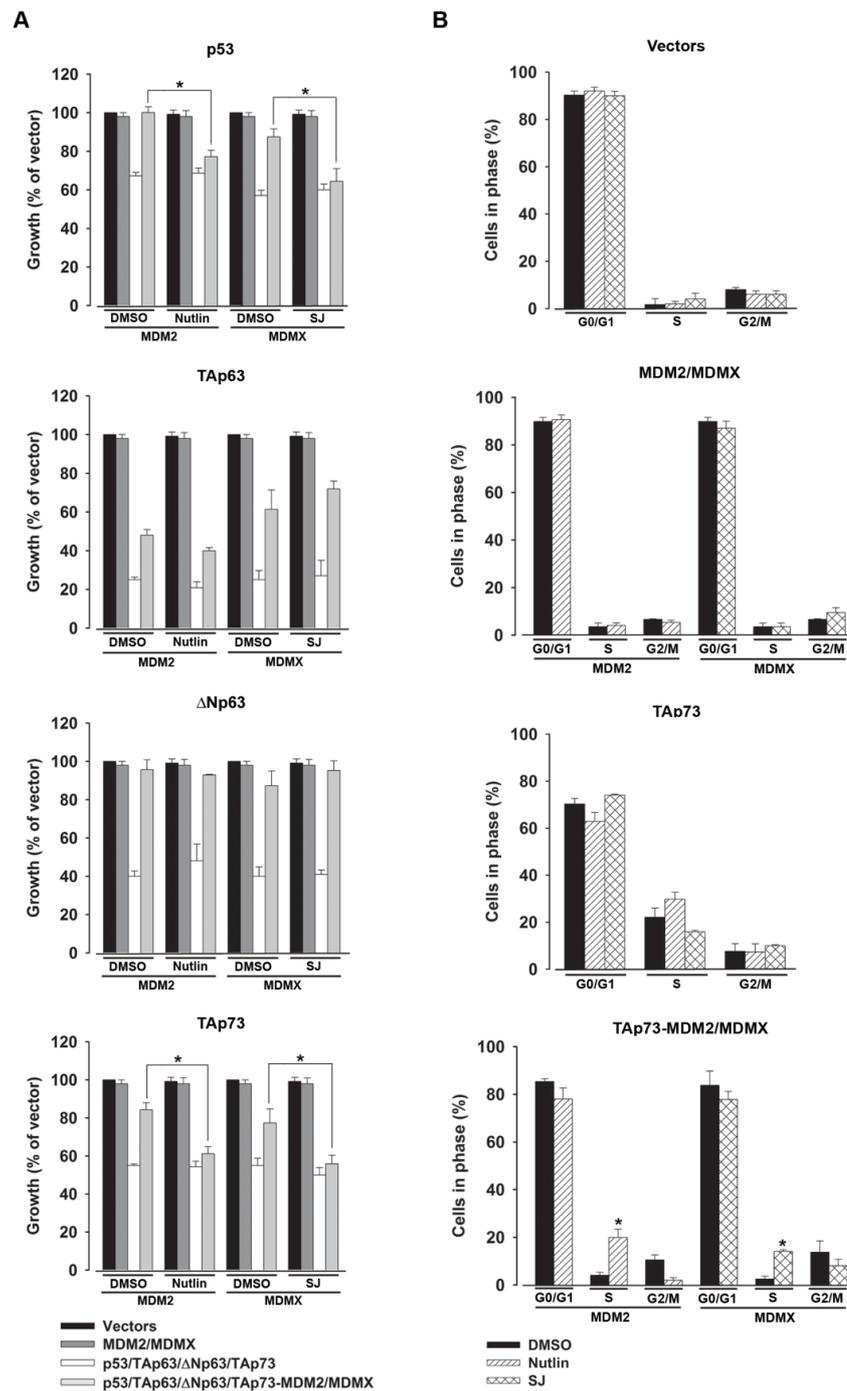


Figure 32. Nutlin-3a and SJ-172550 revert the inhibitory effect of MDM2 and MDMX, respectively, on TAp73, but not on TAp63 and ΔNp63. Yeast cells co-expressing human TAp63, ΔNp63 or TAp73 and/or human MDM2/X, and control yeast (vectors) were grown in induction selective medium with 10 μM nutlin-3a, 20 μM SJ-172550 or DMSO only until the control yeast reached 0.4 OD₆₀₀. **(A)** Effect of nutlin-3a and SJ-172550 on the inhibitory activity of MDM2 and MDMX, respectively, on TAp63-, ΔNp63- and TAp73-induced yeast growth inhibition. Results are plotted setting as 100% growth the number of CFU obtained with control yeast incubated with

DMSO only. Data are mean \pm S.E.M. of five independent experiments; values significantly different are indicated ($*P < 0.05$). (B) Effect of nutlin-3a and SJ-172550 on the inhibitory activity of MDM2 and MDMX, respectively, on TAp73-induced cell cycle arrest. Data are mean \pm S.E.M. of two independent experiments; values significantly higher than DMSO only ($*P < 0.05$).

It must be noted that for the concentration range tested, the maximal release of MDM2 and MDMX inhibitory effects on TAp73 (and p53), without cytotoxicity on control yeast, was achieved at 10 μ M nutlin-3a and 20 μ M SJ-172550, respectively. In fact, in yeast cells co-expressing TAp73 and MDM2, the TAp73-induced growth inhibition/cell cycle arrest was almost completely re-established in the presence of 10 μ M nutlin-3a. Similar results were obtained with yeast cells co-expressing TAp73 and MDMX treated with 20 μ M SJ-172550 (Fig. 32A, B).

3.4.5. Discussion

In light of the high complexity of the p53 family pathway in human cells, each assay able to functionally characterize p53 family proteins is expected to contribute to the understanding of their biology and to define their potential drugability. This concept prompted us to develop yeast-based assays to study, in a well-defined genetic background, some aspects regarding the biology of the p53 family members. In particular, we applied, for the first time, to p63 α (TA and Δ N isoforms) and p73 α (TA isoform) the yeast growth inhibition assay that we previously developed for p53. The p63 and p73 proteins were expressed in yeast, where their effects on cell proliferation and death were analysed. In general, p53, TAp63, Δ Np63 and TAp73 exhibited similar activities on yeast cell proliferation and death. In fact, as previously reported for p53 (Coutinho et al., 2009b), also TAp63, Δ Np63 and TAp73 induced yeast growth inhibition associated with cell cycle arrest. In the present work, it was shown that this growth inhibitory effect of p53 family proteins in yeast was also associated with the induction of cell death. Intriguingly, as previously observed by our group with p53 (Coutinho et al., 2011), also TAp63, Δ Np63 and TAp73, when expressed in yeast, neither induced apoptosis nor stimulated the apoptosis induced by H₂O₂ and acetic acid, two well-known yeast apoptotic stimuli [reviewed in (Pereira et al., 2008)]. On the contrary, p53, TAp63, Δ Np63 and TAp73 not only stimulated the rapamycin-induced autophagic cell death, but also triggered *per se* an autophagic cell death in yeast. It is worth to highlight the different sensitivity to the autophagosome pathway detected among the p53 family proteins. In fact, while a mild sensitivity to the autophagosome machinery was detected with TAp63, the truncated

isoform Δ Np63 showed to be highly sensitive to the lack of *ATG* genes. It must be noted that despite the results obtained in the present work, the induction of an apoptotic cell death by p53-overexpressing yeast cells has been also reported by other authors (Hadj Amor et al., 2008; Palermo et al., 2013). The distinct experimental conditions used in these works (*i.e.* culture media) may explain such differences, since they may interfere with the mitochondrial response. In fact, evidence has been provided showing an intimate correlation between the p53-mediated apoptotic cell death in yeast and the mitochondrial response, particularly with the mitochondrial protein Nuc1p (homologous of mammalian EndoG nuclease) (Palermo et al., 2013). In that work (Palermo et al., 2013), it was shown that *NUC1*-deficient cells were resistant to p53-induced apoptotic cell death. We may therefore speculate that, for some reason, the Nuc1p mitochondrial pathway is inhibited in our experimental conditions leading to an inactivation of the apoptotic pathway and to the consequent activation of an alternative mechanism of cell death triggered by p53.

Currently, the crucial role of p53, p63 and p73 in the direct and indirect modulation of autophagy is widely recognized (Napoli and Flores, 2013). However, several aspects of the complex molecular mechanisms underlying the regulation of autophagy by p53 family proteins and the subsequent functional consequences are still not completely characterized (Napoli and Flores, 2013). Notably, the role of p63 and p73 in autophagy under unstressed conditions was recently pointed out as a relevant issue that, however, requires further clarification (Napoli and Flores, 2013). The present work describes, for the first time, the induction of an autophagic cell death by p53 family proteins in unstressed yeast cells. Consistent with our results in yeast, it was observed an association of p53-mediated autophagy with *ATG1* and *ATG5* (Crighton et al., 2006; Gao et al., 2011), which were recently identified as direct transcriptional p53 target genes in human cells (Napoli and Flores, 2013). Moreover, in mammals, it was recently demonstrated that TAp73 can regulate autophagy through transcriptional activation of the *ATG5* gene (He et al., 2013). The results herein obtained, showing that *ATG1* and *ATG5* are necessary for autophagy induced by p53 family proteins in yeast, are in agreement with previous data from human cells (Kenzelmann Broz et al., 2013) that showed that *ATG1* and *ATG5* are induced not only by p53 but also by other members of the p53 family. Moreover, although the stimulation of autophagy by full length p63 and p73 was already described in human cells, the role of the truncated forms lacking the TA domain in this biological process is still unclear (Napoli and Flores, 2013). The results from yeast reveal that, like the full length proteins, the truncated forms, particularly Δ Np63, may also trigger an autophagic cell death. Accordingly, it was recently shown the induction of autophagy-related genes in cisplatin-treated human cells overexpressing Δ Np63 α (Huang et al., 2012).

The induction of autophagy by p53, TAp63, Δ Np63 and TAp73, observed in this study, appears to be associated with ROS generation. A link between the oxidative state of yeast cells and the p53 toxicity was previously highlighted (Pearson and Merrill, 1998; Hadj Amor et al., 2008). Consistently with our results, a correlation between ROS generation and the stimulation of an autophagic cell death has been reported in distinct human tumour cells (Chen et al., 2009). Furthermore, it was recently shown that silibinin, a p53 activator, induced an autophagic cell death in human fibrosarcoma HT1080 cells via ROS generation (Duan et al., 2011).

The effect of TAp63, Δ Np63 and TAp73 on actin depolarization and protein expression levels was also studied for the first time in yeast. The results showed that, as formerly observed with p53 (Chapter 3.3.), also TAp63, Δ Np63 and TAp73 induced actin depolarization. Although a much lower degree of actin depolarization was observed with TAp63, Δ Np63 and TAp73, when compared to p53, these results point to an involvement of TAp63, Δ Np63 and TAp73 on actin cytoskeleton organization. Additionally, as reported for p53 (Chapter 3.3.), also TAp63, Δ Np63 and TAp73 increased the actin expression levels, an effect inhibited by MDM2 and MDMX. These results indicate that *ACT1*, recently identified as a putative yeast p53 target gene (Chapter 3.3.), likely represent a putative TAp63, Δ Np63 and TAp73 target gene in yeast. A direct regulation of actin expression levels by p53 (Comer et al., 1998) and p63 (Osada et al., 2005) was already described in human cells. The results obtained expressing TAp73 in yeast suggest that actin may also be a transcriptional target of TAp73 in human cells. Although possible indirect effects cannot be discarded at this stage, this finding may open the way to the development of simplified yeast p53 family transactivation assays based on the analysis of the expression levels of actin as an endogenous p53 family target gene. These assays may be used for the analysis of the impact of cofactors and small molecules on the transcriptional activity of these proteins.

It has been described that, like p53, the TA isoforms of p63 and p73 are transcriptionally active proteins, thus performing similar roles to p53 in mammalian cells. In fact, these proteins are well-known by their anti-proliferative activities in mammalian cells, which is consistent with the results obtained in yeast. However, since Δ N isoforms lack the N-terminal TA domain, it has been proposed that these isoforms are transcriptionally inactive (Yang et al., 1998). In fact, Δ N isoforms are generally described as dominant-negative regulators of TA isoforms, being often associated with the promotion of cell proliferation [reviewed in (Alsafadi et al., 2009)]. In spite of this, in the present work, the full-length and truncated forms of p63 exhibited similar effects in yeast, suggesting that Δ Np63 was also transcriptionally active. Actually, very recently, the

intrinsic ability in transactivation specificity of TAp63a and Δ Np63a was investigated in depth using yeast (Monti et al., 2014). It turned out that both isoforms are transcriptionally active, but exhibit intrinsic differences in transactivation specificities that depend on distinct features of DNA target sites. Interestingly, these differences in transactivation specificities were not observed with the corresponding p73 or p53 proteins. These results in yeast have been further supported by previous studies reporting that the Δ Np63 α isoform in tumour cells can also bind and regulate p53-target genes inducing cell cycle arrest and apoptosis (Dohn et al., 2001; Wu et al., 2003).

The regulation of p53 family proteins by MDM2 and MDMX is still a controversial issue in human cells, particularly for p63. In the present study, it was shown that, as observed for p53 (Chapters 3.1. and 3.3.), MDM2 and MDMX negatively regulate the activity of TAp63, Δ Np63 and TAp73 in yeast. Moreover, no significant differences were observed between the potencies of MDM2 and MDMX towards a same p53 family protein. However, differences were observed with the inhibitory effect of MDM2 and MDMX among the different p53 family proteins. In fact, in the growth assays, while a weak inhibitory effect of the MDMs, particularly of MDM2, was observed on TAp63, a quite strong effect of the MDMs was observed on the truncated isoform Δ Np63. Concerning TAp63, our results are consistent with those obtained in human cells by Kadakia and colleagues (2001) (Kadakia et al., 2001): MDM2 and MDMX were able to inhibit the TAp63 transcriptional activity. In a recent *in vitro* study, it was demonstrated that both MDM2 and MDMX bind, although weakly, to the TA domain of p63 (Zdzalik et al., 2010). Nevertheless, other authors reported the absence of interaction between TAp63 and the MDM proteins, both in human tumour cells (Little and Jochemsen, 2001; Wang et al., 2001) and in a Y2H (Kojima et al., 2001). The slight effect of the MDMs on TAp63 may be the basis of the controversial results around this issue. The interactions between Δ Np63 and MDMs are even less understood in human cells. In fact, although it was recently reported that MDM2 binds to Δ Np63 α in human cell (Galli et al., 2010), to our knowledge, no evidence of interaction between Δ Np63 and MDMX has been reported. Concerning the interaction of TAp73 with MDMs, the results herein presented are consistent with those obtained in human cells. It was shown that both MDM2 (Bálint et al., 1999; Wang et al., 2001) and MDMX (Ongkeko et al., 1999; Wang et al., 2001) bind to TAp73, inhibiting its transcriptional activity. More recently, *in vitro* studies also confirmed the interaction of MDM2 and MDMX with TAp73 (Zdzalik et al., 2010).

With the reconstitution in yeast of the human regulatory networks involving the p53 family proteins and MDM2/MDMX, simplified yeast-based assays were developed to screen for inhibitors of the interactions described above. These assays can be easily

adapted to a large scale screening based on simple measurements of the yeast cell growth, speeding up the discovery of inhibitors of these interactions, as observed with the p53-MDM2 interaction (Chapter 3.1.). Additionally, by using the developed assays, the effect of nutlin-3a and SJ-172550 on the interaction of TAp63, Δ Np63 and TAp73 with MDM2/MDMX was also studied, in this work. Interestingly, although nutlin-3a and SJ-172550 did not interfere with the negative effect of MDM2/MDMX on TAp63 and Δ Np63, these compounds revealed to be potential inhibitors of the TAp73 interaction with MDM2/MDMX. Notably, in human tumour cells, it was demonstrated that nutlin-3 inhibits the binding between TAp73 α and MDM2 (Lau et al., 2008). However, to our knowledge, this is the first report of the effect of SJ-172550 on the p73-MDMX interaction.

Chapter 3.5.

LEM2: a small molecule activator of the p73 pathway by potential MDM2 inhibition

Based on the previous identification of pyranoxanthone **1** as the first inhibitor of the p53-MDM2 with a xanthone scaffold (Chapter 3.1.), it was hypothesized if the derivative of the pyranoxanthone **1**, called LEM2 (Fig. 33), could also act as an inhibitor of this interaction. The non-selectivity of LEM2 to the p53 pathway led us, in the present study, to the identification of a putative inhibitor of the p73-MDM2 interaction.

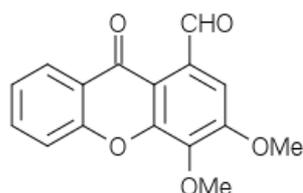


Figure 33. Chemical structure of 1-carbaldehyde-3,4-dimethoxy-9H-xanthen-9-one (LEM2).

3.5.1. Identification of LEM2 as potential inhibitor of the p53-MDM2 interaction using the yeast approach

The effect of LEM2 as inhibitor of the p53-MDM2 interaction was tested using the previously developed yeast assay to search for inhibitors of this interaction (Chapter 3.1.). The effect of 1-100 μ M LEM2 was compared to that obtained with the positive control (nutlin-3a). From the dose-response curves obtained (data not shown), the concentration of 10 μ M was selected as the lowest concentration at which a significant reversion of the MDM2 negative impact on p53 activity was obtained. Similarly to nutlin-3a, when yeast cells co-expressing p53 and MDM2 were treated with LEM2 for 42 h, approximately 92% of the p53-induced yeast growth inhibition was re-established, without interfering with the activity of p53 or MDM2 when expressed alone (Fig. 34).

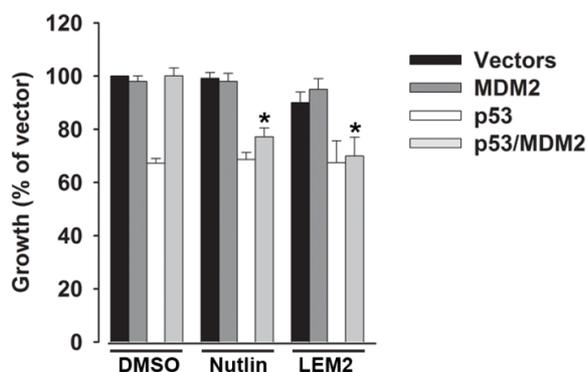


Figure 34. LEM2 reverts the inhibitory effect of MDM2 on p53-induced growth inhibition. Control yeast and yeast cells expressing only p53 or MDM2, and co-expressing p53 and MDM2 were incubated with 10 μ M nutlin-3a, 10 μ M LEM2 or DMSO only, for 42 h. Nutlin-3a was used as

positive control. Results were estimated considering 100% growth, the number of CFU obtained with control yeast (empty vectors). Data are mean \pm S.E.M. of five independent experiments; values obtained with yeast co-expressing p53 and MDM2 treated with the compound significantly different from DMSO only (* $P < 0.05$).

3.5.2. LEM2 acts through a p53-independent mechanism in human tumour cell lines

In order to analyse the tumour cell growth inhibitory effect of LEM2 and the involvement of the p53 pathway in its activity, human colon adenocarcinoma HCT116 cell lines with wt p53 (HCT116 p53^{+/+}) and its p53-null isogenic derivative (HCT116 p53^{-/-}) were used. The GI₅₀ values obtained for LEM2 in p53^{+/+} and p53^{-/-} HCT116 cells indicated that this small molecule has a potent growth inhibitory effect against these tumour cells (Table 6). However, contrary to nutlin-3a, no significant differences in the growth inhibitory effect of LEM2 were observed between p53^{+/+} and p53^{-/-} HCT116 cells (Table 6).

Table 6. GI₅₀ values obtained for LEM2 in human colon adenocarcinoma HCT116 tumour cell lines.

Compounds	GI ₅₀ (μM) ^a	
	HCT116 p53 ^{+/+}	HCT116 p53 ^{-/-}
Nutlin-3A	3.67 \pm 0.34	24.0 \pm 0.7*
LEM2	1.28 \pm 0.01	1.44 \pm 0.02

^aThe GI₅₀ was determined for all tested compounds after 48 h incubation, using the SRB assay. Cells were exposed to serial dilution of nutlin-3a (from 0.62 to 50 μM) or LEM2 (from 0.12 to 10 μM). Data are mean \pm S.E.M. of three independent experiments. Values significantly different from HCT116 p53^{+/+} (* $P < 0.05$).

In addition, as observed in HCT116 p53^{+/+} cells (data not shown), also in HCT116 p53^{-/-} cells, the growth inhibitory effect of LEM2, at GI₅₀ concentration, was associated with the induction of G2/M-phase cell cycle arrest (Fig. 35A), early apoptosis (Fig. 35B) and PARP cleavage (Fig. 35C).

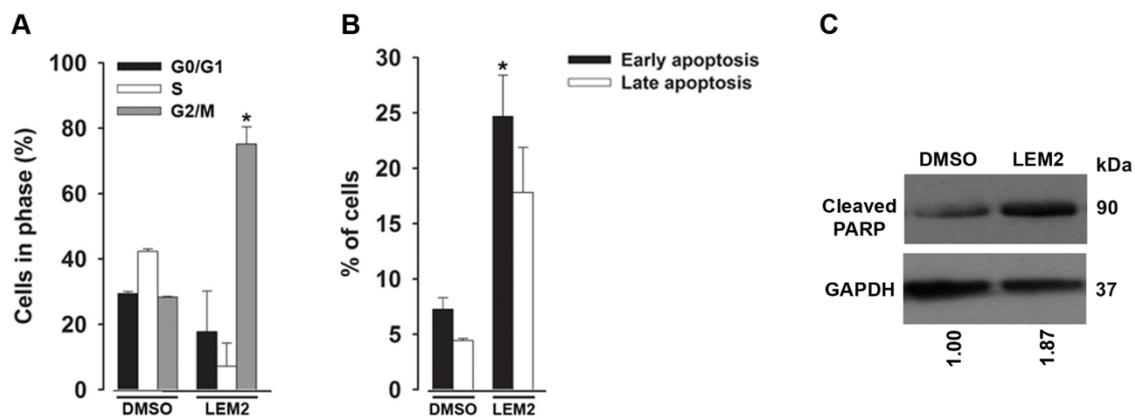


Figure 35. LEM2 induces cell cycle arrest and apoptosis in HCT116 p53^{-/-} cells. (A) LEM2 induced G2/M-phase cell cycle arrest in HCT116 p53^{-/-} cells. The effect was determined after 24 h treatment with 1.28 μM (GI₅₀) of LEM2; cell cycle phases were analysed by flow cytometry using PI; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (**P* < 0.05). (B) LEM2 induces early apoptosis in HCT116 p53^{-/-} cells. The effect was determined after 48 h treatment with 1.28 μM LEM2. Apoptosis was analysed by flow cytometry using FITC-Annexin V and PI; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (**P* < 0.05). (C) LEM2 leads to PARP cleavage in HCT116 p53^{-/-} cells. Western blot analysis was performed after 48 h treatments with 1.28 μM LEM2 or DMSO only; immunoblot represent one of two independent experiments; GAPDH was used as loading control; band intensities were normalized against the control sample (DMSO), which was set as 1.

Altogether, the obtained results showed that LEM2 had a p53-independent *in vitro* antitumor activity.

3.5.3. Identification of LEM2 as potential inhibitor of the p73-MDM2 interaction using the yeast approach

Based on the results obtained above, which showed that LEM2 was not a selective activator of the p53 pathway, and since HCT116 p53^{-/-} tumour cells are also p63-null (Lau et al., 2008) [also confirmed by Western blot analysis (data not shown)], it was hypothesized if LEM2 could interfere with the p73 pathway. To address this issue, the previously developed yeast-based assay to search for inhibitors of the p73-MDM2 interaction (Chapter 3.4) was used. In this yeast assay, although the expression of human MDM2 does not interfere with the yeast cell growth and cell cycle progression, its co-

expression with p73 significantly reduces the p73-induced yeast growth inhibition and cell cycle arrest (Fig. 36A-B). Similarly to nutlin-3a (Chapter 3.4.; Fig. 36A-B), when yeast cells co-expressing p73 and MDM2 were treated with 10 μ M LEM2 for 40 h, approximately 98% and 72% of the p73-induced growth inhibition and S-phase cell cycle arrest, respectively, were reestablished, without interfering with the activity of p73 or MDM2 when expressed alone (Fig. 36A-B).

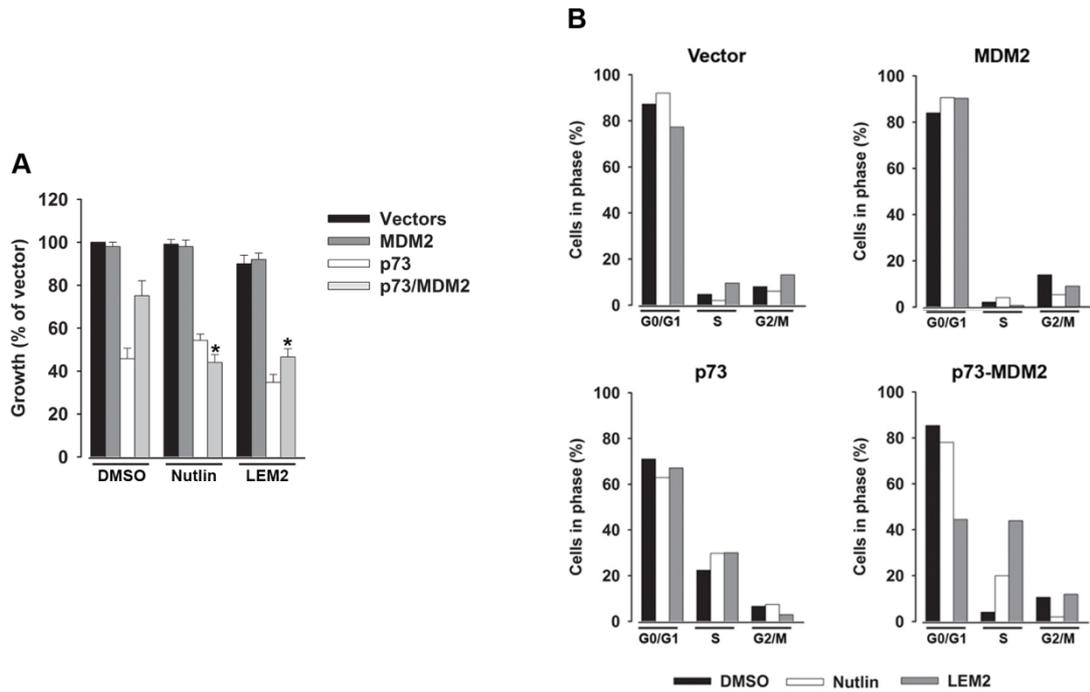


Figure 36. LEM2 reduces the negative effect of MDM2 on p73 activity in yeast. Yeast cells were incubated with 10 μ M nutlin-3a, 10 μ M LEM2 or DMSO only, for 40 h. Nutlin-3a was used as positive control. **(A)** Effects of 10 μ M nutlin-3a and 10 μ M LEM2 on the growth of control yeast, yeast expressing only p73 or MDM2, and yeast co-expressing p73 and MDM2. Results were estimated considering 100% growth, the number of CFU obtained with control yeast (empty vectors). Data are mean \pm S.E.M. of five independent experiments; values obtained with yeast co-expressing p73 and MDM2 treated with the compound significantly different from DMSO only (* P < 0.05). **(B)** Effects of 10 μ M nutlin-3a and 10 μ M LEM2 on the yeast cell cycle progression of control yeast, yeast expressing only p73 or MDM2, and yeast co-expressing p73 and MDM2. Yeast cell cycle phases were quantified by flow cytometry; data represent one of two independent experiments.

3.5.4. LEM2 activates the p73 pathway in human tumour cells by potential MDM2 inhibition

Based on the exposed above, the molecular mechanism of action of LEM2 associated with its growth inhibitory effect in HCT116 p53^{-/-} tumour cell lines was investigated. To assess the activation of the p73 pathway by LEM2, the protein expression levels of p73 and of p73 target genes, such as MDM2, p21 and Bax, were checked by Western blot analysis in HCT116 p53^{-/-} tumour cells (Fig. 37). The results obtained showed an increase of the p73 baseline levels upon treatment with 1.28 μM LEM2 (Fig. 37). In addition, 1.28 μM LEM2 increased the protein levels of the p73 transcriptional targets MDM2, p21 and Bax (Fig. 37), what indicates the reduction of the p73 inhibition by MDM2 with the promotion of its transcriptional activity.

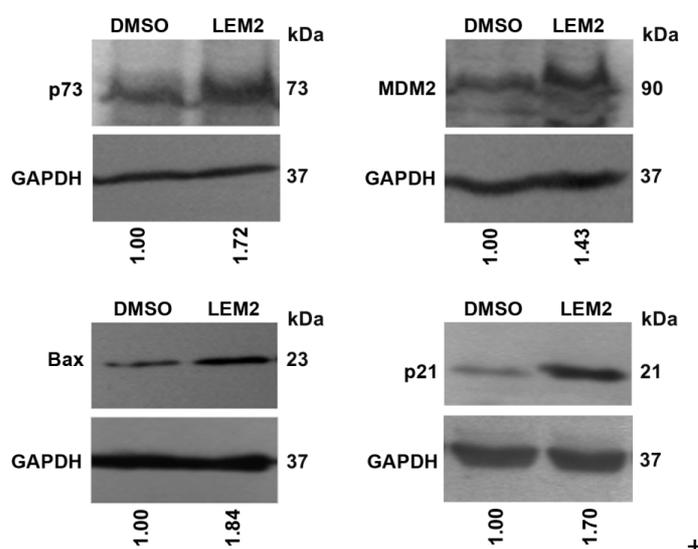


Figure 37. LEM2 increases the expression levels of p73 and of p73 target genes (MDM2, p21 and Bax) in HCT116 p53^{-/-} tumour cells. Western blot analysis was performed for HCT116 p53^{-/-} tumour cells after 8 h (p21), 16 h (p73, Bax) and 48 h (MDM2) treatment with the GI₅₀ concentration of LEM2 (1.28 μM) or DMSO only. Immunoblots represent one of two independent experiments; GAPDH was used as loading control; band intensities were normalized against the control sample (DMSO), which was set as 1.

3.5.5. Discussion

Likewise p53, the complex p73 function is tightly regulated by MDM2 [reviewed in (Collavin et al., 2010)]. As such, the activation of the p73 tumour suppressive activities through inhibition of its interaction with MDM2 represents an important and appealing anticancer therapeutic strategy, particularly against tumours with p53-null or with mutant p53 [reviewed in (Bisso et al., 2011)]. In spite of this, to date, only one inhibitor of the p73-MDM2 interaction, nutlin-3a, was reported (Lau et al., 2008). In fact, it was shown that

nutlin-3a, a well-known inhibitor of the p53-MDM2 interaction, was also able to inhibit the p73-MDM2 interaction, causing p73 accumulation and inducing the expression of p73 transcription targets in tumour cells without p53 (Lau et al., 2008). Similarly to nutlin-3a, LEM2 also led to p73 stabilization and to the up-regulation of p73 transcriptional targets, such as MDM2, p21 and Bax in p53- and p63-null HCT116 cells.

Altogether, in this work, a potential inhibitor of the p73-MDM2 interaction was identified, which may pave the way to the structure-based design of new p73-MDM2 interaction inhibitors with improved pharmacological properties. Great therapeutic applications can be envisaged for these compounds particularly against tumour cells with null or mutant p53.

Further work is still underway in our laboratory in order to confirm the effect of LEM2 on the p73-MDM2 interaction. Particularly, co-immunoprecipitation assays will be performed, in human tumour cells, in order to confirm the disruption of the p73-MDM2 interaction by LEM2. Additionally, to prove the activation of a p73-dependent pathway, by LEM2, this protein will be silenced by iRNA technique in tumour cells. Finally, since mitochondrially-targeted agents have been highlighted by their efficiency against chemotherapy-refractory cancer cells (Fulda et al., 2010), the induction of a mitochondria apoptotic pathway by LEM2 will be also checked.

Chapter 4

General discussion
Future perspectives

The contribution of “humanized yeast systems” to our current understanding of human disease-associated proteins, particularly in the identification of their regulators, has been undeniable [reviewed in (Pereira et al., 2012a; Guaragnella et al., 2014)].

As crucial regulators of cell proliferation and death, the p53 family proteins have been recognized as attractive therapeutic targets in cancer treatment [reviewed in (Wei et al., 2012)]. In spite of this, the biology of these proteins remains not completely clarified [reviewed in (Vilgelm et al., 2008)]. Additionally, small molecule modulators of the activity of p53 family proteins, particularly of p63 and p73, with improved pharmacological properties are highly required [reviewed in (Chung and Irwin, 2010)]. Hence, with the present thesis, it was intended to exploit the yeast cell system for biological and pharmacological studies of p53 family proteins. An important outcome of this thesis was the validation of the efficacy of the developed yeast assays in the discovery of modulators of p53 family activity with potential anticancer properties.

4.1. DEVELOPMENT OF YEAST ASSAYS FOR BIOLOGICAL AND PHARMACOLOGICAL STUDIES OF P53 FAMILY PROTEINS

In a previous work from our group, it was shown that the expression of wt p53 in yeast induced a marked growth inhibition associated with S-phase cell cycle arrest (Coutinho et al., 2009b). That work opened the way to the exploitation of the yeast system in the study of other members of the p53 family, particularly of p63 (TA and ΔN isoforms) and p73 (TA isoform), as well as of their regulation by MDM2 and MDMX.

The works performed, in this thesis, showed similarities among the activities of the different p53 family proteins expressed in yeast. In fact, all of them induced growth inhibition associated with S-phase cell cycle arrest, ROS generation, increase of actin expression levels and depolarization, as well as cell death neither through apoptosis nor necrosis, but through autophagy. However, besides these similarities, member- or even isoform-specific effects are “highlighted” by our experimental approaches. Different sensitivities to the autophagosome pathway, to the inhibitory effect of MDM2 and MDMX, and to small molecule modulators of their interactions with MDM2/MDMX clearly emerged. Altogether, highlighted similarities and differences support the sensitivity and specificity of these yeast-based systems for studying p53 family members. The results obtained may contribute, in a joint effort with more complex systems, to a deeper understanding of the molecular mechanism underlying the function and regulatory networks of these “multitasking” p53 family members. Most importantly, the developed yeast assays may contribute to the elucidation of additional aspects of the biology of p53 family proteins,

particularly in autophagy, and to study the impact of cofactors and small molecules on their activities (Fig. 38).

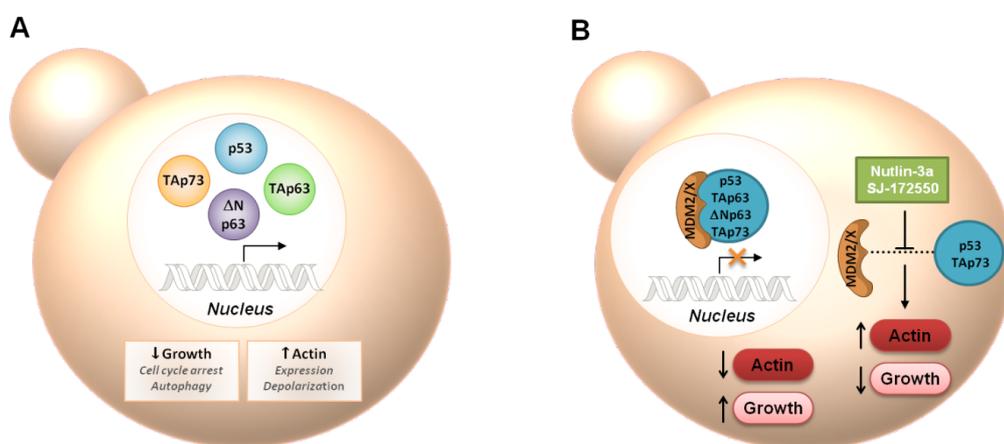


Figure 38. Novel yeast target-directed screening assays developed for biological and pharmacological studies of p53 family proteins: Yeast growth-inhibitory (analysis of cell growth) and transactivation (analysis of actin protein expression levels) assays. (A) Yeast p53 family assays: p53 family proteins, individually expressed in yeast, are localized in the nucleus and induce growth inhibition associated with cell cycle arrest, autophagy, increase of actin depolarization and protein expression levels. **(B) Yeast p53 family-MDMs assays:** co-expression of MDM2/MDMX inhibits the growth inhibition and increase of actin protein levels induced by individual p53 family proteins; the inhibitory effect of MDM2/MDMX on p53 and TAp73 activity is reverted by nutlin-3a (for MDM2) and SJ-172550 (for MDMX).

4.2. IDENTIFICATION OF NEW SMALL MOLECULE MODULATORS OF P53 FAMILY PROTEINS USING THE DEVELOPED YEAST TARGET-DIRECTED SCREENING ASSAYS

It was a major objective of this thesis to use the developed yeast assays in the search for small molecule modulators of p53 family proteins. Based on this, under the scope of this thesis, the developed yeast assays led us to the identification of:

- i)* The first inhibitor of the p53-MDM2 interaction with a xanthone scaffold, the pyranoxanthone 1 (LEM1) [International Patent Request (Inga et al., 2013)];
- ii)* α -Mangostin and gambogic acid as potential inhibitors of the p53-MDM2 interaction, what contributed to the elucidation of the molecular mechanism of

action the two well-known natural xanthone derivatives with potent antitumor properties;

- iii) A potential inhibitor of the p73-MDM2 interaction (LEM2);
- iv) SJ-172550 as a potential inhibitor of the p73-MDMX interaction.

Besides the outcomes achieved in this thesis, with the yeast systems here developed other promising small molecule modulators of the p53 family proteins have already been identified in our laboratory, which molecular mechanisms have been validated in human tumour cells, particularly:

- i) The first inhibitor of the p53-MDM2 interaction with a oxazoloisoindolinone scaffold, the compound 3a (Soares et al., 2014);
- ii) A dual inhibitor of the p53-MDM2/MDMX interaction, the tryptophanol-derived oxazolopiperidone lactam OXAZ-1 (*Work submitted for publication*).

Oxazoloisoindolinone 3a and OXAZ-1 exhibited a p53-dependent *in vitro* antitumor activity through induction of cell cycle arrest and apoptosis, p53 stabilization and up-regulation of p53 target genes. The ability of oxazoloisoindolinone 3a and OXAZ-1 to block the p53-MDMs interaction was further demonstrated in human tumour cells by co-immunoprecipitation assays, and molecular docking analysis revealed the putative binding of these compounds to MDM2 (oxazoloisoindolinone 3a) and to MDM2/MDMX (OXAZ-1).

A schematic illustration of the new small molecule modulators of p53 family proteins, identified using the yeast assays developed in this thesis, is presented in Figure 39. These compounds opened the way to a new class of activators of p53 family proteins. Further optimizations of these compounds may be the basis of potential anticancer agents with therapeutic applications. Additionally, they are an incontestable proof of concept for the effectiveness of this cell system in the discovery of regulators of the activity of p53 family proteins.

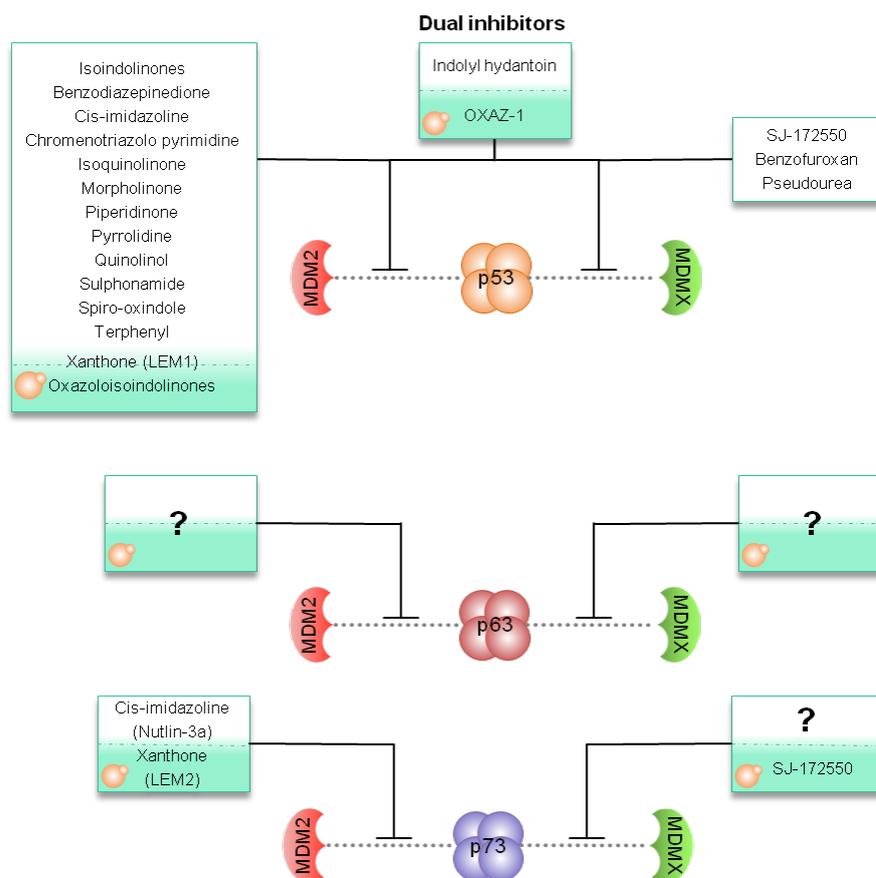


Figure 39. New small molecule modulators of the activity of p53 family proteins emerged from the developed yeast target-directed screening assays. (? : Unknown compounds)

4.3. FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

In the present work, the yeast cell model was used for an independent analysis of p53, p63 (full length and truncated forms) and p73 functionality. Several relevant insights about the biology and “potential” druggability of these human proteins are revealed for the first time in yeast. Additionally, new yeast target-directed approaches are provided for future studies of the p53 family network and for the identification of new regulators of the activity of p53 family proteins. These assays have proved to be highly effective as first-line assays, before studies in more complex cell systems.

This thesis therefore highlights new challenges and opportunities that may emerge from the use of yeast as a model organism and powerful system in the study of p53 family proteins. Particularly, it is anticipated that, for the near future, the use of these yeast assays holds great promise in the discovery of novel anticancer agents with therapeutic applicability. Beyond that, in a strict relation with the other outcomes emerged from this thesis, it would be interesting to:

- i)* Undertake the hit-to-lead optimization of LEM1 and LEM2, in order to obtain new small molecules with improved pharmacological properties;
- ii)* Develop similar yeast assays for other relevant p63/p73 isoforms in tumorigenesis (such as β and γ isoforms), in order to study their roles in cell proliferation and death, their transcriptional activities and regulation by MDM2/MDMX;
- iii)* Elucidate the effect of ΔN on TA proteins and to search for modulators of ΔN -TA interactions, since ΔN proteins can function as inhibitors of p53, TAp63 and TAp73;
- iv)* Develop *in vitro* binding assays for a deeper analysis of the molecular mechanism of compounds by defining their interactions with the target proteins of the p53 family pathway.

Chapter 5

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Chapter 6

Appendix

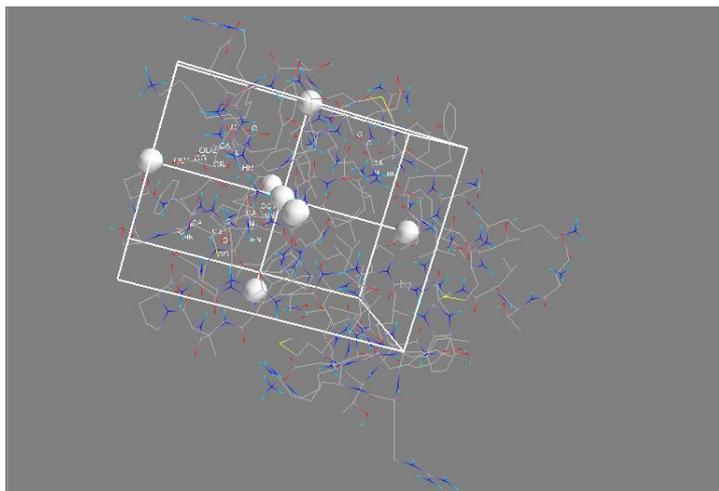


Figure A1. Grid dimensions and position in the MDM2 in PyRxAutodockVina.

Table A1. Results of docking simulations for MDM2 (with grid box) performed in PyRx/AutoDockVina.

Ligand	Binding affinity (kcal.mol ⁻¹)
3,4-dihydro-12-hydroxy-2,2-dimethyl-2 <i>H</i> ,6 <i>H</i> -pyrano[3,2- <i>b</i>]xanthen-6-one (1)	-7.6
2,3-dihydro-10-hydroxy-3,3-dimethyl-11-(2-methylbut-3-en-2-yl)pyrano[2,3- <i>c</i>]xanthen-7(1 <i>H</i>)-one (12)	-7.3
5-hydroxy-2,2-dimethylpyrano[3,2- <i>b</i>]xanthen-6(2 <i>H</i>)-one	-7.3
6-hydroxy-3,3,5-trimethylpyrano[2,3- <i>c</i>]xanthen-7(3 <i>H</i>)-one	-7.2
6-hydroxy-3,3-dimethylpyrano[2,3- <i>c</i>]xanthen-7(3 <i>H</i>)-one	-7.1
3,4-dihydro-5-hydroxy-2,2-dimethylpyrano[3,2- <i>b</i>]xanthen-6(2 <i>H</i>)-one	-7.0
2,3-dihydro-4-hydroxy-2,3,3-trimethylfuro[3,2- <i>b</i>]xanthen-5-one	-7.0
2,3-dihydro-6-hydroxy-3,3-dimethylpyrano[2,3- <i>c</i>]xanthen-7(1 <i>H</i>)-one	-6.9
(4-chlorophenyl)[3-(4-chlorophenyl)-7-iodo-2,5-dioxo-1,2,3,5-tetrahydro-4 <i>H</i> -1,4-benzodiazepin-4-yl]acetic acid (bzd)*	-6.9
3-(3-methylbut-2-enyloxy)-9 <i>H</i> -xanthen-9-one	-6.8
3-(3-methylbut-2-enyloxy)-1-hydroxy-9 <i>H</i> -xanthen-9-one	-6.7
2,3-dihydro-6-hydroxy-3,3,5-trimethylpyrano[2,3- <i>c</i>]xanthen-7(1 <i>H</i>)-one (14)	-6.7
(4-(<i>tert</i> -pentyloxy)-2-hydroxyphenyl)(3,4-dihydro-5-hydroxy-2,2-dimethyl-2 <i>H</i> -chromen-6-yl)methanone	-6.6
3,4- <i>bis</i> (3-methylbut-2-enyloxy)-9 <i>H</i> -xanthen-9-one (2)	-6.6
3-(3-methylbut-2-enyloxy)-4-hydroxy-9 <i>H</i> -xanthen-9-one (3)	-6.5
3,4-dihydro-5-hydroxy-2,2-dimethyl-12-(3-methylbut-2-enyl)pyrano[3,2- <i>b</i>]xanthen-6(2 <i>H</i>)-one	-6.4
2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-[1,4]dioxino[2,3- <i>a</i>]xanthen-12-one	-6.1
4-hydroxy-3-methoxy-9 <i>H</i> -xanthen-9-one	-6.1
2-(((<i>S</i>)-oxiran-2-yl)methoxy)-1-hydroxy-9 <i>H</i> -xanthen-9-one (10)	-6.0
9-oxo-9 <i>H</i> -xanthen-3,6-diyl disulfate	-6.0
1,2-dihydro-5-hydroxy-1,1,2,4-tetramethylfuro[2,3- <i>c</i>]xanthen-6-one	-6.1
3-(3-methylbut-2-enyloxy)-1-hydroxy-2-methyl-9 <i>H</i> -xanthen-9-one	-6.1
4-hydroxy-1-(isobutylamino)-3-methoxy-9 <i>H</i> -xanthen-9-one (13)	-6.0
2,3-dimethoxy-9 <i>H</i> -xanthen-9-one	-5.9
3,4-dihydroxy-9 <i>H</i> -xanthen-9-one (6)	-5.9
4-hydroxy-9 <i>H</i> -xanthen-9-one	-5.9
1,7-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.9
1,3-dihydroxy-2-methyl-9 <i>H</i> -xanthen-9-one (11)	-5.9
5,8,10-trihydroxy-7 <i>H</i> -benzo[<i>c</i>]xanthen-7-one	-5.9
3-(3-methylbut-2-enyloxy)-1-hydroxy-4-(3-methylbut-2-enyl)-9 <i>H</i> -xanthen-9-one	-5.9
1-(6-bromohexyloxy)-9 <i>H</i> -xanthen-9-one (5)	-5.9
2-methoxy-9 <i>H</i> -xanthen-9-one	-5.8
3-methoxy-9 <i>H</i> -xanthen-9-one	-5.8
(±)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-11 <i>H</i> -1,4-dioxino[2,3- <i>b</i>]xanthen-11-one (4)	-5.8
1-hydroxy-9 <i>H</i> -xanthen-9-one	-5.8
3-(6-bromohexyloxy)-4-hydroxy-9 <i>H</i> -xanthen-9-one	-5.8
3,5-dimethoxy-9 <i>H</i> -xanthen-9-one	-5.7
4-methoxy-9 <i>H</i> -xanthen-9-one	-5.7

3-hydroxy-9 <i>H</i> -xanthen-9-one	-5.7
9 <i>H</i> -xanthen-9-one	-5.7
1-carbaldehyde-4-hydroxy-3-methoxy-9 <i>H</i> -xanthen-9-one (8)	-5.6
3,4- <i>bis</i> (6-bromohexyloxy)-9 <i>H</i> -xanthen-9-one	-5.6
1-methoxy-9 <i>H</i> -xanthen-9-one	-5.6
2,3-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.6
2-hydroxy-9 <i>H</i> -xanthen-9-one	-5.6
1,3-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.6
3-(3-methylbut-2-enyloxy)-1-hydroxy-2-methyl-4-(3-methylbut-2-enyl)-9 <i>H</i> -xanthen-9-one	-5.6
nutlin-3a*	-5.6
2,2',4,4'-tetracetylbenzophenone (9)	-5.5
3,6-dihydroxy-9 <i>H</i> -xanthen-9-one (7)	-5.5
3-hydroxy-5-methoxy-9 <i>H</i> -xanthen-9-one	-5.5
1,2-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.5
3,4-dimethoxy-9 <i>H</i> -xanthen-9-one	-5.4
3-hydroxy-4-methoxy-9 <i>H</i> -xanthen-9-one	-5.4
1,2-dimethoxy-9 <i>H</i> -xanthen-9-one	-5.4
(±)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-[1,4]dioxino[2,3- <i>c</i>]xanthen-7-one	-5.3
2-glucopyranose-9-oxo-9 <i>H</i> -xanthen-1,3,6,7-tetrayl tetraacetate	-4.3
varacin**	-4.2

*Positive controls

**Negative control

Table A2. Results of docking simulations for MDM2 (total protein) done in PyRx/AutoDockVina.

Ligand	Binding affinity (kcal.mol ⁻¹)
3-(3-methylbut-2-enyloxy)-1-hydroxy-4-(3-methylbut-2-enyl)-9 <i>H</i> -xanthen-9-one	-8.0
(±)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-11 <i>H</i> -1,4-dioxino[2,3- <i>b</i>]xanthen-11-one (4)	-7.9
3-(3-methylbut-2-enyloxy)-1-hydroxy-2-methyl-4-(3-methylbut-2-enyl)-9 <i>H</i> -xanthen-9-one	-7.9
3,4-dihydro-5-hydroxy-2,2-dimethyl-12-(3-methylbut-2-enyl)pyrano[3,2- <i>b</i>]xanthen-6(2 <i>H</i>)-one	-7.9
(4-(<i>tert</i> -pentyloxy)-2-hydroxyphenyl)(3,4-dihydro-5-hydroxy-2,2-dimethyl-2 <i>H</i> -chromen-6-yl)methanone	-7.8
(±)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-[1,4]dioxino[2,3- <i>c</i>]xanthen-7-one	-7.7
3,4-dihydro-12-hydroxy-2,2-dimethyl-2 <i>H</i> ,6 <i>H</i> -pyrano[3,2- <i>b</i>]xanthen-6-one (1)	-7.6
3,4- <i>bis</i> (3-methylbut-2-enyloxy)-9 <i>H</i> -xanthen-9-one (3)	-7.6
2,3-dihydro-10-hydroxy-3,3-dimethyl-11-(2-methylbut-3-en-2-yl)pyrano[2,3- <i>c</i>]xanthen-7(1 <i>H</i>)-one (12)	-7.6
5-hydroxy-2,2-dimethylpyrano[3,2- <i>b</i>]xanthen-6(2 <i>H</i>)-one	-7.5
nutlin-3a*	-7.4
1,2-dihydro-5-hydroxy-1,1,2,4-tetramethylfuro[2,3- <i>c</i>]xanthen-6-one	-7.4
6-hydroxy-3,3,5-trimethylpyrano[2,3- <i>c</i>]xanthen-7(3 <i>H</i>)-one	-7.4
3,4-dihydro-5-hydroxy-2,2-dimethylpyrano[3,2- <i>b</i>]xanthen-6(2 <i>H</i>)-one	-7.3

2,3-dihydro-6-hydroxy-3,3-dimethylpyrano[2,3-c]xanthen-7(1 <i>H</i>)-one	-7.3
6-hydroxy-3,3-dimethylpyrano[2,3-c]xanthen-7(3 <i>H</i>)-one	-7.3
2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-[1,4]dioxino[2,3-a]xanthen-12-one	-7.2
2,3-dihydro-6-hydroxy-3,3,5-trimethylpyrano[2,3-c]xanthen-7(1 <i>H</i>)-one (14)	-7.2
3-(3-methylbut-2-enyloxy)-9 <i>H</i> -xanthen-9-one	-7.1
2,3-dihydro-4-hydroxy-2,3,3-trimethylfuro[3,2-b]xanthen-5-one	-7.1
3-(3-methylbut-2-enyloxy)-4-hydroxy-9 <i>H</i> -xanthen-9-one (2)	-7.0
3-(3-methylbut-2-enyloxy)-1-hydroxy-9 <i>H</i> -xanthen-9-one	-7.0
2,2',4,4'-tetracetylbenzophenone (9)	-6.9
(4-chlorophenyl)[3-(4-chlorophenyl)-7-iodo-2,5-dioxo-1,2,3,5-tetrahydro-4 <i>H</i> -1,4-benzodiazepin-4-yl]acetic acid (bzd)*	-6.9
3-(3-methylbut-2-enyloxy)-1-hydroxy-2-methyl-9 <i>H</i> -xanthen-9-one	-6.9
5,8,10-trihydroxy-7 <i>H</i> -benzo[<i>c</i>]xanthen-7-one	-6.8
9-oxo-9 <i>H</i> -xanthene-3,6-diyl disulfate	-6.6
4-hydroxy-1-(isobutylamino)-3-methoxy-9 <i>H</i> -xanthen-9-one (13)	-6.6
1-(6-bromohexyloxy)-9 <i>H</i> -xanthen-9-one (5)	-6.5
4-hydroxy-3-methoxy-9 <i>H</i> -xanthen-9-one	-6.2
2-(((<i>S</i>)-oxiran-2-yl)methoxy)-1-hydroxy-9 <i>H</i> -xanthen-9-one (10)	-6.3
3-(6-bromohexyloxy)-4-hydroxy-9 <i>H</i> -xanthen-9-one	-6.2
4-methoxy-9 <i>H</i> -xanthen-9-one	-6.1
2-glucopyranose-9-oxo-9 <i>H</i> -xanthene-1,3,6,7-tetrayl tetraacetate	-6.1
3,4-bis(6-bromohexyloxy)-9 <i>H</i> -xanthen-9-one	-6.1
2,3-dimethoxy-9 <i>H</i> -xanthen-9-one	-6.0
3,4-dihydroxy-9 <i>H</i> -xanthen-9-one (6)	-6.0
3,5-dimethoxy-9 <i>H</i> -xanthen-9-one	-6.0
3-hydroxy-4-methoxy-9 <i>H</i> -xanthen-9-one	-6.0
1,3-dihydroxy-2-methyl-9 <i>H</i> -xanthen-9-one (11)	-6.0
1-formyl-4-hydroxy-3-methoxy-9 <i>H</i> -xanthen-9-one (8)	-5.9
1,7-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.9
1-methoxy-9 <i>H</i> -xanthen-9-one	-5.9
2-methoxy-9 <i>H</i> -xanthen-9-one	-5.9
3,4-dimethoxy-9 <i>H</i> -xanthen-9-one	-5.9
3-methoxy-9 <i>H</i> -xanthen-9-one	-5.9
4-hydroxy-9 <i>H</i> -xanthen-9-one	-5.9
1-hydroxy-9 <i>H</i> -xanthen-9-one	-5.9
1,2-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.8
2-hydroxy-9 <i>H</i> -xanthen-9-one	-5.8
3-hydroxy-5-methoxy-9 <i>H</i> -xanthen-9-one	-5.8
3-hydroxy-9 <i>H</i> -xanthen-9-one	-5.8
1,3-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.8
1,2-dimethoxy-9 <i>H</i> -xanthen-9-one	-5.7
2,3-dihydroxy-9 <i>H</i> -xanthen-9-one	-5.7
3,6-dihydroxy-9 <i>H</i> -xanthen-9-one (7)	-5.7
9 <i>H</i> -xanthen-9-one	-5.7
varacin**	-4.5

*Positive control
 **Negative control

Table A3. Results of docking stimulations for MDM2 (PDB code: 1YCR) performed with PyRx/AutoDockVina.

α-mangostin (15)				gambogic acid (16)			
pose number	binding affinity (kcal.mol ⁻¹)	RMSD upper bound ^a	RMSD lower bound ^b	pose number	binding affinity (kcal.mol ⁻¹)	RMSD upper bound ^a	RMSD lower bound ^b
1	-6.8	0	0	1	-7.8	0	0
2	-6.8	8.604	2.12	2	-7.7	2.09	1.095
3	-6.6	7.71	4.144	3	-7.1	9.61	4.982
4	-6.5	3.535	1.973	4	-7.0	4.522	2.157
5	-6.4	9.326	5.869	5	-6.7	8.035	2.245
6	-6.4	8.722	5.55	6	-6.6	2.226	1.491
7	-6.2	9.576	5.601	7	-6.6	7.873	4.49
8	-6.2	4.767	2.187	8	-6.4	9.409	4.889
9	-6.2	8.919	4.583	9	-6.1	7.848	2.171
nutlin-3a				varacin			
pose number	binding affinity (kcal.mol ⁻¹)	RMSD upper bound ^a	RMSD lower bound ^b	pose number	binding affinity (kcal.mol ⁻¹)	RMSD upper bound ^a	RMSD lower bound ^b
1	-7.4	0	0	1	-4.5	0	0
2	-7.4	7.956	4.548	2	-4.5	4.423	3.087
3	-6.5	7.135	4.844	3	-4.2	3.973	2.921
4	-6.5	6.975	4.047	4	-4.2	4.535	2.999
5	-6.3	6.039	3.665	5	-4.0	4.451	3.372
6	-6.3	8.243	3.723	6	-3.9	4.456	3.208
7	-6.3	3.898	2.662	7	-3.8	5.747	4.218
8	-6.2	5.558	3.756	8	-3.7	10.156	8.904
9	-6.1	2.745	1.959	9	-3.6	5.317	4.315

RMSD = root mean square deviation; conformations with lower RMSD values are more similar to the best docked conformation. ^aRMSD/upper bound matches each atom in one conformation with itself in the other conformation, ignoring any symmetry. ^bRMSD/lower bound compares equivalent atoms (the closest atom of the same element type) between conformations.

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