

Seeking optimized TAp73-targeting agents for neuroblastoma treatment

Inês Ferreira da Mota

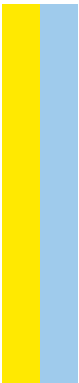
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Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Abstract

Neuroblastoma (NB) is one of the most prevalent childhood solid tumours, with 90% of cases arising in children under ten years old. This paediatric cancer is characterized by a high complexity and diversity, showing the patients diagnosed at an advanced stage poor prognosis and low overall survival rates. In addition, NB therapy is commonly associated with drug resistance and toxic side effects. The limited efficiency of the available therapies against NB makes urgent the search for more effective and safer therapeutic strategies.

The tumour suppressor TAp73, which belongs to the p53 family proteins, has important functions, namely in neuronal differentiation. The inhibition of TAp73 by endogenous modulators, such as MDM2 and mutp53, seems to be related to NB, especially in high-risk and/or relapsed cases. Thus, a promising and beneficial strategy for the treatment of NB would involve the activation of TAp73.

In previous work, our group revealed that the xanthone LEM2 was able to activate TAp73 and consequently, to restore its tumour suppressor activity by disrupting the interaction of TAp73 with MDM2 and mutp53; however, the low aqueous solubility of this compound limited the *in vivo* studies. Thus, an analogue of LEM2, the 1-(dibromomethyl)-3,4,6-trimethoxy-9H-xanthen-9-one (LEM3), was obtained.

In this thesis, the molecular mechanism of action of LEM3, as a potential activator of TAp73, and its antitumour activity, were studied. LEM3 demonstrated potent growth inhibitory activity, associated with cell cycle arrest and apoptosis, in NB cells. It also interfered with the expression levels of several transcriptional targets of TAp73 and decreased poor prognostic markers in NB. Additionally, LEM3 was able to inhibit TAp73-MDM2 and TAp73-mutp53 interactions in yeast and human tumour cells. The interference of LEM3 with several microRNAs involved in TAp73 pathway was also evaluated. However, it was found that LEM3 did not regulate p73-related miRNAs.

In conclusion, the results herein obtained support that LEM3 is an activator of TAp73 with promising applications in the treatment of NB.

Keywords: microRNAs; neuroblastoma; TAp73; targeted anticancer therapy; xanthenes

Resumo

O neuroblastoma (NB) é um dos tumores sólidos infantis mais predominantes, com 90% dos casos surgindo em crianças com menos de dez anos de idade. Este cancro pediátrico é caracterizado por uma elevada complexidade e diversidade, apresentando os pacientes diagnosticados numa fase avançada um mau prognóstico clínico e baixas taxas de sobrevivência global. Além disso, a terapia do NB está normalmente associada a resistências à terapêutica e a efeitos colaterais tóxicos. A eficácia limitada das terapias disponíveis contra o NB torna urgente a procura de estratégias terapêuticas mais eficazes e seguras.

O supressor tumoral TAp73, que pertence à família das proteínas p53, tem funções importantes, nomeadamente na diferenciação neuronal. A inibição da TAp73 por moduladores endógenos, como MDM2 e mutp53, parece estar relacionada com o NB, especialmente em casos de alto risco e/ou recidivas. Assim, uma estratégia promissora e benéfica para o tratamento do NB pode envolver a activação da TAp73.

Num trabalho anterior, o nosso grupo demonstrou que a xantona LEM2 era capaz de activar TAp73 e, conseqüentemente, restaurar a sua actividade supressora tumoral, através da inibição da interacção de TAp73 com MDM2 ou mutp53; contudo, a baixa solubilidade aquosa deste composto limitou os estudos *in vivo*. Assim, foi obtido um análogo de LEM2, 1-(dibromomethyl)-3,4,6-trimethoxy-9H-xanthen-9-one (LEM3).

Nesta tese, estudou-se o mecanismo molecular de acção do composto LEM3, como um potencial activador da TAp73, bem como a sua actividade antitumoral. O LEM3 demonstrou uma potente atividade inibitória do crescimento, associada à paragem do ciclo celular e à apoptose, em células de NB. Também interferiu com os níveis de expressão de vários alvos transcripcionais da TAp73 e diminuiu os marcadores de mau prognóstico do NB. Adicionalmente, o LEM3 foi capaz de inibir as interacções TAp73-MDM2 e TAp73-mutp53 em células de leveduras e células humanas tumorais. Adicionalmente, avaliou-se também a interferência do LEM3 com vários microRNAs envolvidos com a via da TAp73. No entanto, verificou-se que o LEM3 não regulava os miRNAs relacionados com esta proteína.

Em conclusão, os resultados aqui obtidos apoiam que o LEM3 é um activador da TAp73 com aplicações promissoras no tratamento do NB.

Palavras-chave: microRNAs; neuroblastoma; TAp73; terapia anticancerígena direccionada; xantonas

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Abbreviations

AD - Acidic domain

AGO - Argonaute

ALK - Anaplastic lymphoma kinase

AKT - Protein kinase B

ASCT - Autologous stem cell transplantation

ATCC - American Type Culture Collection

ATRX - alpha-thalassemia/mental retardation, X-linked

AURKA - Aurora A kinase

BAX - Bcl-2 associated X protein

BCA - Bicinchoninic acid

Bcl-2 - B-cell lymphoma 2

BDNF - Brain-derived neurotrophic factor

BET - Bromodomain and extra-terminal

BSA - Bovine serum albumin

cDNA - Complementary DNA

CFU - Colony-forming unit

CHIP - Carboxyl terminus of Hsc70-interacting protein

CNVs - Copy number variations

Co-IP - Co-immunoprecipitation

Cq - Cycle of quantification

DBD - DNA-binding domain

DEPC - Diethylpyrocarbonate

DFMO - Difluoromethylornithine

DGCR8 - DiGeorge Syndrome Critical Region 8

DN - Dominant-negative

DMSO - Dimethyl sulfoxide

EMT - Epithelium-mesenchymal transition

FBS - Fetal bovine serum

FGF-2 - Fibroblast growth factor 2

GADD45 - Growth arrest and DNA-damage inducible protein

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GI₅₀ - Half-maximal cell growth inhibitory concentration

GOF - Gain-of-function

HDAC - Histone deacetylase
HDC - High dose chemotherapy
HSP - Heat shock protein
IC - Induction chemotherapy
IDRFs - Image-defined risk factors
IL-8 - Interleukin 8
IMCT - Intensive multimodal clinical therapy
IgG - Immunoglobulin G
INRG - International Neuroblastoma Risk Group
INRGSS - International Neuroblastoma Risk Group Staging System
INSS - International Neuroblastoma Staging System
LIN28B - Lin-28 homolog B
LMO1 - LIM domain only 1
LOH - Loss of heterozygosity
MAPK - Mitogen-activated protein kinase
MDM2 - Murine double minute 2
MDMX - Murine double minute X
MDR1 - Multidrug resistance 1
miRNA - MicroRNA
mRNA - Messenger RNA
mTOR - Mammalian target of rapamycin
MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mutp53 - Mutant p53
MYCN - Gene encoding N-MYC
NB - Neuroblastoma
NES - Nuclear export signal
NGF - Nerve growth factor
NLS - Nuclear localization signal
nm - Nanometers
NRAS - Neuroblastoma RAS viral oncogene homolog
nt - Nucleotides
OD - Oligomerization domain
OD₆₀₀ - Optical density at 600 nm
PARP-1 - Poly (ADP-ribose) polymerase-1

PBS - Phosphate-buffered saline
PHOX2B - Paired-like homeobox 2B
PI - Propidium iodide
PI3K - Phosphoinositide 3-kinase
PRD - Proline-rich domain
pre-miRNA - Precursor miRNA
pri-miRNA - Primary miRNA
PUMA - p53 upregulated modulator of apoptosis
qPCR - Quantitative Polymerase Chain Reaction
RanGTP - GTP-binding nuclear protein Ran
RFD - Ring-finger domain
RISC - RNA-induced silencing complex
RITA - Reactivation of p53 and induction of tumour cell apoptosis
RNA Pol II - RNA polymerase II
RNase - Ribonuclease
rpm - Rotations per minute
SAM - Sterile- α motif
SDS-PAGE - Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM - Standard error of the mean
shRNAs - Endogenous short hairpin RNAs
snoRNAs - Small nucleolar RNAs
SV40 - Simian virus T antigen
TAD - Amino-terminal transactivation domain
TAp73 - Full-length p73 (with the transactivation domain)
TBS-T - Tris-buffered saline with Tween 20
TERT - Telomerase reverse transcriptase
Trk - Tropomyosin receptor kinase
UTR - Untranslated region
VEGF - Vascular endothelial growth factor
wt - Wild-type
XPO5 - Exportin-5
ZFD - Zinc-finger domain
 Δ Np73 - N-terminal truncated p73

1. INTRODUCTION

1.1 General concepts about cancer

Neoplasm is a lesion characterized by the disordered proliferation of cells in the body. When it forms an abnormal mass of tissue it is often referred to as a "tumour" ¹. According to the potential to cause harm to the patient, neoplasms can be classified as benign or malignant (cancer) and also, neoplasms with intermediate malignancy/"borderline" ¹. The categorisation of a neoplasm is fundamental for the correct clinical and therapeutic approach.

Carcinogenesis, also called tumourigenesis, is the process through which the normal cell acquires the characteristics of the neoplastic cell. Tumourigenesis is the result of genetic and epigenetic alterations in normal cells caused by endogenous or exogenous agents (carcinogens), which are not completely repaired ²⁻⁵. In general, there are three groups of genes involved in tumourigenesis: inducer genes (proto-oncogenes), inhibitor genes (tumour suppressor genes), and apoptosis regulator genes (important in controlling the proliferation of abnormal cells). Therefore, the accumulation of genomic injuries leads to the activation of oncogenes (which result from the activation of proto-oncogenes) and the inactivation of tumour suppressor genes ^{3, 4}.

In most malignant neoplasms it is possible to identify four phases of development: transformation, growth, local invasion, and metastatisation. These four phases are only observed in malignant neoplasms. The transformation process gives the affected cells a set of characteristics that allow their autonomous uncontrolled multiplication. Moreover, it is a process that takes place over time, requiring the accumulation of successive alterations that confer a selective advantage to the neoplastic cell ^{2, 5}.

During the last decades, several fundamental properties of malignant neoplastic cells have been identified and are now known as hallmarks of cancer (Figure 1). Hallmarks of cancer were presented as a set of acquired capabilities necessary to form malignant tumours. In 2000, the first six hallmarks were proposed and since then more cellular characteristics have emerged, with fourteen currently described ^{6, 7} (Figure 1).

The concept of hallmarks is useful for a better understanding of the complexity of cancer biology and also to explain the difficulty in cancer treatment since the presence of all these described tumour characteristics contributes to aggressive tumour phenotypes. The difficulty in the treatment is related to the need for the therapeutic perspective to reach several hallmarks and not only one.

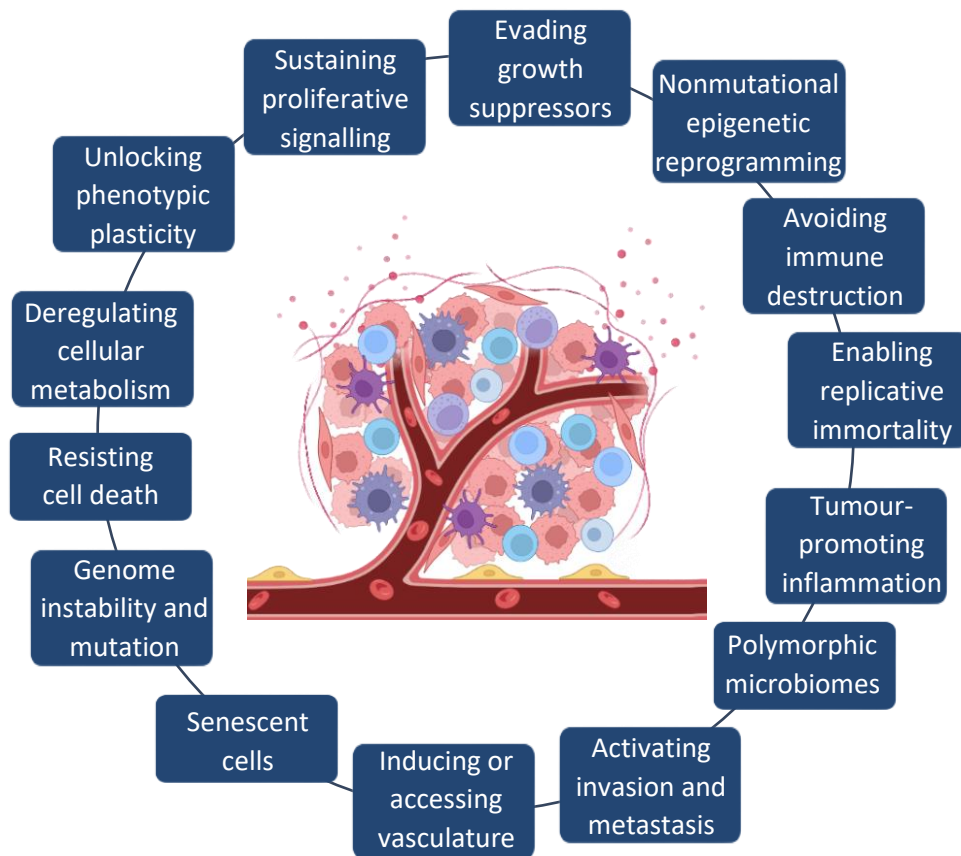


Figure 1. Hallmarks of cancer. Adapted from ⁷.

Transitions between epithelium and mesenchyme are evolutionarily conserved processes, initially described in the context of embryonic development. Epithelial cells have barrier and secretion functions, while mesenchymal cells present support functions, with a relevant role in tissue repair, also having the ability to move and acquire other morphologies. In addition to the existence of the epithelial and mesenchymal phenotype, there may exist an intermediate state (hybrid phase of the two/phase of epithelium-mesenchymal transition (EMT)), a more variable morphology and, from the molecular point of view, a decrease in epithelial markers and an increase in markers for mesenchymal expression. This happens because for the EMT to occur, the cells have to modify themselves. This is a progressive and dynamic process, in which the cell has to lose its epithelial characteristics and gain mesenchymal characteristics ^{5, 7}.

In cancer, EMT is involved in the acquisition of intratumoral cell heterogeneity, tissue invasion, metastasis, acquisition of resistance to therapy, among others ⁵.

1.2 Neuroblastoma

1.2.1 Epidemiology and aetiology

Neuroblastoma (NB) is the most common extracranial solid tumour arising in the peripheral sympathetic nervous system, accounting for around 10% of all paediatric cancer deaths ⁸. The incidence of NB is approximately 10.5 per million children aged 0-14 years in North America and Europe, with minimal ethnic or geographical variability ^{9, 10}.

The likelihood of developing NB varies by age, with the highest number of cases detected in newborns and young children, and decreasing over the first 10 years. Although NB rarely occurs in adolescents and young adults, it tends to be more indolent but lethal when NB develops in this group of patients. NB is more frequent in boys than in girls ^{8, 11}.

The underlying aetiology of most NB tumours is unknown, although exposures during conception and pregnancy are under investigation ^{9, 10, 12}. Hence, further studies are needed in identifying risk factors related to the pathogenesis and development of these tumours. These malignant tumours usually occur sporadically, but familial cases have been reported with a prevalence of approximately 1-2%. Most patients with familial NB have germline mutations in the anaplastic lymphoma kinase (*ALK*) or paired-like homeobox 2B (*PHOX2B*) genes. In the case of sporadic tumours up to 15% of cases also result from mutations in *ALK*. Additionally, common transformation mutations include polymorphisms in the lin-28 homolog B (*LIN28B*) gene and amplification of *MYCN* oncogene ^{9, 10}.

1.2.2 Clinical characteristics and staging

NB tumours can be classified as embryonic neuroendocrine tumours because evidence suggests that they originate from neural crest progenitor cells during fetal development ¹⁰. During embryonic development, the neural crest cells develop and migrate to differentiate into several different lineages, but in the case of NB, it occurs only from the precursor cells or stem cells of the sympathoadrenal lineage ^{11, 13}. Most NB tumours arise in the adrenal medulla and the rest develop along the sympathetic nervous system chain from the neck to the pelvis ^{11, 14}.

NB is a complex disease that presents biological, clinical, morphological, and genetic heterogeneity. The clinical presentation of this disease can range from a localized disease with spontaneous regression or differentiation of the tumour, associated with a good prognosis, to a high-risk aggressive progression with metastasis ^{14, 15}.

The signs and symptoms of this disease are extremely variable and depend on the age at diagnosis, the site of the primary tumour, the stage of the disease, and occasionally on the associated paraneoplastic syndromes, such as Horner syndrome, which is associated with local aggressiveness ^{11, 14}.

One of the systems used to classify NB patients is the International Neuroblastoma Staging System (INSS), depending on the degree of surgical excision of the tumour at the time of diagnosis and metastases. Thus, the INSS stratifies into the following stages: 1, 2A/2B, 3, 4, and 4S. Briefly, stages 1 and 2 correspond to distinct localized tumours, stage 3 is identified by an advanced local area disease, and stages 4 or 4S show metastatic tumours. In general, patients with early-stage tumours (stages 1 and 2) have a better prognosis compared to those with advanced tumours (stages 3 and 4). In contrast, infants under the age of one year old always show good outcomes due to spontaneous regression of the tumour, even if they have disseminated disease limited to the liver, skin, or bone marrow (stage 4S) ^{10, 11, 14}.

More recently, the International Neuroblastoma Risk Group (INRG) Task Force proposed a new staging system, the International Neuroblastoma Risk Group Staging System (INRGSS). This system was developed to determine the child's stage and risk group before treatment initiation, based on clinical criteria and image-defined risk factors (IDRFs). The INRGSS classifies NB into various stages, as described in Table 1 ^{11, 14}.

Table 1. International Neuroblastoma Risk Group Staging System (INRGSS).

Tumour Stage	Description
L1	Localized tumour not involving vital structures as defined by IDRFs.
L2	Locoregional tumour with one or more IDRFs.
M	Metastatic tumours (except stage MS tumour).
MS	Metastatic tumours in which metastases are confined to the skin, liver and/or bone marrow in children under 18 months of age. It is equivalent to stage 4S.

Adapted from ¹⁶.

The INRG classification is a newer system based on the INRGSS staging system that includes the IDRFs, as well as the following prognostic variables, such as age at diagnosis, tumour histology, the presence or absence of *MYCN* gene amplification in tumour cells, chromosome 11q status and DNA ploidy. Thus, these markers help to classify each NB into one of four categories: very low, low, intermediate and high risk ^{10, 11}.

The development of genomic technology and the integration of next-generation sequencing may include more genetic abnormalities, which will allow better clarification of risk groups, translating into more personalised treatment ¹¹.

1.2.2.1. Spontaneous regression of neuroblastoma

Spontaneous regression of cancer can be defined as the partial or complete disappearance of primary or metastatic disease in the absence of treatment or in the presence of therapy, which has no influence on the disease ¹⁷.

Although spontaneous regression is an uncommon occurrence, NB is the disease in which it may occur more frequently. Furthermore, several studies have indicated that the number of patients with tumours in spontaneous regression without clinical detection is equivalent to the amount of NB cases that have been detected ¹⁷.

This phenomenon presents an undefined aetiology, although several mechanisms have been proposed in an attempt to understand how spontaneous regression occurs in NB, among which, neurotrophin deprivation, loss of telomerase activity, humoral/cellular immunity and alterations in epigenetic regulation ¹⁷.

Considering the staging systems, NB stage 4S or MS is manifested by a high rate of spontaneous regression of the tumour ^{17, 18}. Several studies have described certain key features of stage 4S or MS tumours, including ^{11, 17, 18}:

- Hyperdiploid or near-triploid nuclear DNA content with whole chromosome gains;
- Absence of amplification in the *MYCN* oncogene;
- Low expression of the telomerase reverse transcriptase (*TERT*) gene;
- High levels of tropomyosin receptor kinase A (TrkA);
- High levels of tumour suppressor genes and proteins.

However, spontaneous regression is not limited to this stage of the disease, because regression can occur even in patients with favourable tumours at other stages ¹⁷.

Possible therapeutic strategies to induce regression of NB include the use of TrkA inhibitors and immunological therapy. Currently, clinical trials are underway using Pan-Trk inhibitors (an antibody that recognises the C-terminal domain of Trk-A, Trk-B and Trk-C) ¹⁷.

1.2.3 Genetic alterations

A distinction can be made between patients with NB, based on their karyotype. Thus, there are tumours with a near-diploid or near-triploid nuclear DNA content ^{11, 19}. Near-triploid tumours usually have entire chromosome gains and losses without structural rearrangements. These are often localized, with a good prognosis, and in patients under 1 year of age. While near-diploid tumours appear in patients older than 1 year of age with advanced disease and have genetic aberrations, such as chromosomal loss. Most NB tumours have a nuclear DNA content in the diploid range ^{11, 19}.

Several genetic alterations have been observed in NB, including gene amplifications, polymorphisms, and chromosomal abnormalities.

1.2.3.1. MYCN

The *MYCN* oncogene encodes the transcription factor N-MYC, which is a member of the MYC family of proto-oncogenes, which also included two other closely related genes, *MYC/MYCC* and *MYCL* ¹¹. N-MYC is a transcriptional master regulator that can activate genes that affect cancer hallmarks, such as sustained growth, and repress genes that drive differentiation ¹².

N-MYC expression is primarily confined to the nervous system and mesenchymal tissues during certain embryonic stages and is expressed at a very low level in adult tissues. In addition, N-MYC plays an important role in normal brain development ¹¹. The degree of *MYCN* amplification strongly correlates with advanced tumour stage and disease progression, and is used as a biomarker for risk stratification ^{11, 12}. Hence, amplification of the *MYCN* and upregulation of its expression are important hallmarks in the progression of high-risk NB ²⁰.

Due to the high structural and sequence homology within the MYC family, N-MYC shares partial functional redundancy with C-MYC in the transcriptional regulation of genes involved in various cellular functions such as proliferation, apoptosis, and differentiation ¹¹.

1.2.3.2. *ALK*

In NB, germline mutations in the *ALK* oncogene have been identified as the leading cause in most cases of familial NB. Due to its proximity to *MYCN*, *ALK* can be co-amplified with *MYCN*. Additionally, *ALK* was identified as a direct transcription target of N-MYC ^{11, 12}.

1.2.3.3. *PHOX2B*

The *PHOX2B* is the first genetic predisposition identified in NB. Physiologically, *PHOX2B* functions as a transcription factor involved in the regulation of the differentiation in the sympathetic nervous system. In NB, *PHOX2B* gene mutations result in loss of function of *PHOX2B* ^{11, 13}. In NB cells, *PHOX2B* mutation is linked to RAS/mitogen-activated protein kinase (MAPK) pathway activation. Furthermore, it has been shown that elevated levels of PHOX2B protein appear to correlate with increased expression of *MYCN* and *ALK* in NB cells ¹³.

1.2.3.4. *LIN28B*

Polymorphic alleles within the *LIN28B* locus are strongly involved in the development of high-risk NB and increase the expression of N-MYC via let-7 microRNAs repression in NB ¹¹⁻¹³.

1.2.3.5. *NRAS*

Neuroblastoma RAS viral oncogene homolog (*NRAS*) is a proto-oncogene that belongs to the Ras gene family ²¹. Mutations in *NRAS* and other genes involved in the RAS-MAPK pathway have been associated with relapsed NB tumours and gain-of-function (GOF) mutations have been associated with aggressive behaviour and poor prognosis in NB¹¹.

1.2.3.6. *TERT*

TERT is another essential gene involved in telomere maintenance. *TERT* rearrangements have been found in high-risk NB patients and are highly linked to poor clinical outcomes. Telomere lengthening caused by abnormally active *TERT* could be a key factor in the transformation of the aggressive subtype of NB. *TERT* is a transcriptional target of N-MYC, so *TERT* levels are higher in *MYCN*-amplified NB tumours ^{11, 22}.

1.2.3.7. ATRX

Another factor in the development of NB is the loss of function of the RNA-helicase alpha-thalassemia/mental retardation, X-linked (*ATRX*) due to somatic mutations. These changes are more common in older individuals, and they are thought to be mutually exclusive with *MYCN* amplification ^{11, 22, 23}.

1.2.3.8. Other genomic rearrangements

LIM domain only 1 (*LMO1*) aberrances, raise *LMO1* expression and are strongly linked to advanced disease and poor prognosis ¹¹.

Several polycomb-group protein members have been shown to play a role in the development and progression of NB tumours. N-MYC/C-MYC and E2F-1 are direct targets of the polycomb complex protein Bmi-1. Bmi-1 is extensively expressed in primary NB tumours, and it plays an important role in NB carcinogenesis ¹¹.

TrkA, TrkB, and TrkC are members of the tropomyosin-related kinase (Trk) family. TrkB and its ligand brain-derived neurotrophic factor (BDNF) are highly expressed in *MYCN*-amplified aggressive NB with a bad prognosis. Unlike TrkB, increased TrkA expression is associated with favourable biological characteristics such as younger age at diagnosis, a lower stage, and the absence of *MYCN* amplification. TrkA and its ligand nerve growth factor (NGF) transduce signals to promote the survival and differentiation of NB cells. TrkC is similarly expressed in *MYCN*-unamplified lower-stage NB malignancies ¹¹.

1.2.3.9. Chromosomal copy number variations

Chromosomal copy number variations (CNVs) are the most common genetic event in NB. Some recurrent alterations include allelic loss of 1p, 3p, 4p, 6q, 11q and 14q, and gains of 1q, 2p and 17q ¹¹.

The most well-characterized genetic alteration in NB is an amplification of the *MYCN* proto-oncogene, associated with a highly aggressive behaviour and poor prognosis ^{10, 11, 14, 19}. This amplification is observed in approximately 25 - 35% of patients. However, it is found in 30 - 40% of stage 3 and 4 NB, and in only 5% of localized tumours or stage 4s NB ^{10, 19}.

As for the most common chromosome copy number alteration, 17q gain, it has been shown in over half of NB cases ^{11, 19}. Loss of heterozygosity (LOH) on chromosomes 1p and 11q has been reported in about 20% and 35 - 40% of NB cases, respectively. Of note, 1p loss

and 17q gain are correlated with amplification of the *MYCN* oncogene and in turn, with poor prognosis of patients with NB ¹⁰. Furthermore, several candidate tumour suppressor genes have been identified in the common LOH regions of 1p, including *TP73* and *mir-34a* ^{12, 14}. Hence, the tumour suppressor protein p73 is mapped in the region (1p36), so the loss of this region might be associated with the loss of p73 activity ²⁴.

In addition, loss of 11q is inversely correlated with *MYCN* amplification and is associated with advanced stage, older age, and unfavourable pathology ^{11, 19}. In relapse tumours, the loss of 6q, 11q and 17p are frequently detected ¹¹.

1.2.4. Treatment according to risk classification

Significant advances have been made in treatment strategies for NB based on risk stratification according to the INRG classification system. In the case of being a clinically stable low-risk disease, surgery is the main treatment strategy, and the tumour mass is excised. In addition, these patients have a 5-year survival rate of more than 90% and some infants have a high propensity for spontaneous tumour regression. For the patients in the intermediate-risk group, one of the most widely used options is milder chemotherapy. However, about 60% of patients have a high risk of NB. For these patients, the intensive multimodal clinical therapy (IMCT) comprises three phases of treatment: induction, consolidation, and maintenance. The first phase, induction chemotherapy (IC) aims to shrink the tumour, and also reduce the risk of metastasis with alternating regimens of high dose chemotherapy (HDC) drugs and surgery. Next, the consolidation phase involves the administration of more intense chemotherapy (HDC) with radiotherapy and autologous stem cell transplantation (ASCT). Finally, the maintenance phase includes immunotherapy and retinoid drug treatment ^{10, 13, 25}.

Despite current intensive multimodality therapy, patients diagnosed at an advanced stage have poor clinical prognosis with a low overall survival rate, with a 5-year survival rate of less than 50%. Response to current treatments can range from complete regression to the development of drug resistance and severe toxicity. Hence, efforts must be strengthened to understand tumour biology, and to develop new, more effective, and safer therapeutic strategies to successfully treat patients with NB ^{10, 13, 25}.

1.2.5. Approaches to targeted therapies for neuroblastoma

New therapeutic approaches have been introduced based on extensive research into the aetiology of NB. There are several preclinical and clinical trials, some of which are still

ongoing, to evaluate the effects of new compounds in patients and to study the potential of compounds targeting particular molecules or signalling pathways of relevance in NB treatment ^{13, 26, 27}.

1.2.5.1. MYCN inhibitors

As previously mentioned, *MYCN* is a therapeutic target for high-risk NB. As such, several alternative strategies have been proposed for inhibiting *MYCN* function. However, to date, there are still no compounds that directly inhibit N-MYC ^{11, 13, 26}.

An example of a therapeutic strategy of inhibition of *MYCN* function is by using the compounds 10058-F4 and 10074-G5, which blocked heterodimerisation (*MYCN* forms heterodimers with MAX protein and therefore causes NB progression) and led to apoptosis of *MYCN*-amplified NB cells. However, *in vivo* assays with these compounds had limitations ^{13, 26}.

Regarding indirect *MYCN* inhibition, one therapeutic strategy is the inhibition of Aurora A kinase (*AURKA*), which is responsible for stabilising *MYCN* and protecting it from proteasomal degradation. In preclinical studies, inhibition of *AURKA* by MLN8237 (alisertib) caused *MYCN* degradation in NB cells ^{11, 13, 26}. Additionally, some clinical trials are ongoing to investigate the combination of *AURKA* inhibitors with chemotherapeutic agents, namely irinotecan and temozolomide ¹¹. Another example that fits this therapeutic alternative is the inhibition of the bromodomain and extra-terminal domain (BET) family proteins. BET proteins affect *MYCN* transcriptional activity. Their inhibition by compounds such as JQ1, OTX015 and GSK1324726A (I-BET726) is another strategy to inhibit *MYCN* expression in NB cells. Clinical trials are underway with these compounds ^{13, 26}. Another alternative related to indirect *MYCN* inhibition is by targeting the phosphatidylinositol 3-kinase/ protein kinase B/ mammalian target of rapamycin (PI3K/AKT/mTOR) pathway because abnormal activation of this pathway is associated with *MYCN* amplification in high-risk NB. Thus, research has been focused on finding inhibitors of PI3K, AKT and mTOR. Several of these agents have been developed, such as SF1126 (inhibitor of PI3K and mTOR) and MK2206 (inhibitor of AKT). Furthermore, the effects of these compounds in combination with chemotherapy are under investigation ^{11, 13}.

1.2.5.2. ALK inhibitors

Several studies have confirmed that *ALK* inhibition is a promising therapeutic approach, considering that *ALK* is expressed on the surface of most NB tumour cells and its inhibition was effective in both wild-type (wt) and mutant *ALK* NB cells ^{27, 28}.

Crizotinib was the first drug to be approved for the treatment of cancers with *ALK* aberrations, acting as an *ALK* inhibitor. In preliminary clinical trials with crizotinib, limited activity was found against the various *ALK* mutants in NB patients ^{11, 28}. Subsequently, clinical trials have been conducted with other *ALK* inhibitors such as lorlatinib, ceritinib, and entrectinib. In addition, entrectinib has also been found promising to act as a TrkB inhibitor, which is associated with poor prognosis in NB, as already mentioned. Preclinical studies have also demonstrated the efficacy of using *ALK* inhibitors, such as lorlatinib, as a single agent and in combination with conventional chemotherapeutic agents ²⁶.

1.2.5.3. *PHOX2B* inhibitors

In preclinical studies, several *PHOX2B* inhibitors have been found to be effective in NB cells. For example, curcumin, diphenylhydantoin and suberoylanilide hydroxamic acid decreased *PHOX2B* expression at messenger RNA (mRNA) and protein levels. A study showed that XAV939 inhibits *PHOX2B* in NB cells, including in SH-SY5Y cells, also sensitizing these cells to treatment when combined with doxorubicin. Another molecule, mycophenolate mofetil, also inhibited *PHOX2B* and induced apoptosis in NB cells ¹³.

1.2.5.4. *LIN28B* inhibitors

Several studies have revealed different compounds potent inhibitors of *LIN28B*. For example, difluoromethylornithine (DFMO) and bortezomib inhibited *LIN28B* in several NB cells. Most importantly, the combination of these two agents caused a greater reduction in the expression levels of this protein ¹³. JQ1 and panobinostat are also compounds capable of reducing *LIN28B*, as well as N-MYC, when in combination therapy, in NB cells ¹³.

1.2.5.5. Angiogenesis inhibitors

Angiogenesis is correlated with tumour growth and metastasis, so tumour vascularity has been associated with aggressive progression in NB patients ^{27, 28}.

The presence of pro-angiogenic molecules is dependent on the stage of the tumour in question ^{13, 28}. For example, vascular endothelial growth factor (VEGF) expression is associated with advanced-stage NB tumours. Furthermore, VEGF165 (an isoform of VEGF) is predominantly expressed in NB cells ^{13, 27}. Thus, the function of angiogenesis inhibitors is to block the formation of new blood vessels and tumour growth. Antiangiogenic agents act on several proteins that have been identified as angiogenic activators, including VEGF, interleukin

8 (IL-8), fibroblast growth factor 2 (FGF-2), among others^{13, 27, 29}. Some preclinical studies with angiogenic inhibitors showed promising results in NB, for example, melatonin inhibited angiogenesis in the NB cell line SH-SY5Y through downregulation of VEGF. Other inhibitors, topotecan and sunitinib decreased VEGF expression in NB cells. Sunitinib also inhibited *MYCN* expression^{13, 28}. Other studies further observed that antiangiogenic therapy has a modest effect when used in monotherapy, being the combination with conventional chemotherapy necessary²⁹.

1.3. Members of the p53 family in cancer

The p53 protein was first identified in 1979 as an oncoprotein that formed a complex with the simian virus T antigen (SV40)^{30, 31}. At that time, p53 was considered an oncogene since its expression levels were higher in cancer cells than in normal cells. However, ten years later, it was discovered that mutations produced overexpression of p53 in cancer cells, and that p53 is a tumour suppressor protein. It was also discovered that p53 regulates the transcription of many genes involved in distinct cellular processes, preventing the multiplication of damaged cells that are more likely to contain mutations and exhibit abnormal cellular growth than undamaged cells. Hence, p53 is widely recognized as "the guardian of the genome" due to its critical function in maintaining genomic integrity and preventing cancer formation^{31, 32}.

The p53 family of transcription factors comprises three genes: *TP53*, *TP63*, and *TP73*, which are located in chromosomes 17p13.1, 3q27-29, and 1p36.2-3 and encode the p53, p63, and p73 proteins, respectively^{33, 34}.

These three members share a high level of structural similarity. Despite this, their functional redundancy is limited, because each protein has its unique roles. For instance, p53 regulates the stress response to suppress tumours, p63 is critical for the correct development of ectodermal-derived tissues, whereas p73 is implicated in the regulation of the nervous and immune systems^{33, 35}.

Each *TP53* family member contains different domains (Figure 2): an N-terminal transactivation domain (TAD), a proline-rich domain (PRD), a DNA-binding domain (DBD), and a C-terminal oligomerization domain (OD)³³. The C-terminal sterile- α motif (SAM) domain is only present in p63 and p73. It is responsible for protein-protein interactions and is found in various proteins involved in developmental regulation^{33, 34}. The DBD has the most homology (63% identity between p53 and p73, and 60% identity between p53 and p63), which explains

how the three proteins may bind to the same DNA sequences and activate the same transcriptional genes, such as those involved in cell cycle arrest and apoptosis ³³.

Of note is that genes of the p53 family members are expressed as several different isoforms, some of which have completely distinct roles in cancer development ³⁶ (Figure 3). Interactions between p53 family members and their isoforms have a significant impact on carcinogenesis and anticancer drug response. The interactions between the ΔN and TA isoforms are among the most researched. Indeed, proteins with an N-terminal acidic TAD, are referred to as TA isoforms (TAp63 and TAp73) and act as tumour suppressors, whereas the N-terminal truncated proteins that lack TAD, are called ΔN isoforms ($\Delta Np63$ and $\Delta Np73$), acting as oncogenes ^{34, 36} (Figure 3).

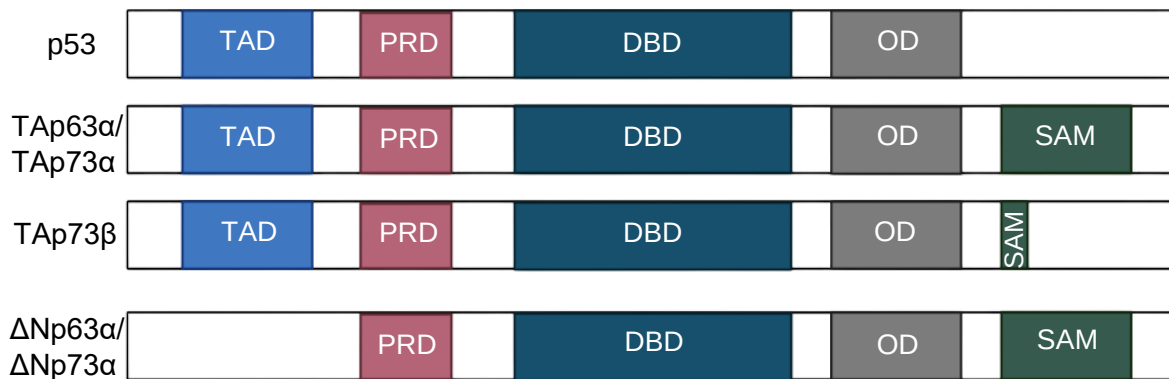


Figure 2. Structural organization of p53 family proteins. TAD: amino-terminal transactivation domain; PRD: proline-rich region; DBD: DNA-binding domain; OD: carboxy-terminal oligomerization domain; SAM: carboxy-terminal sterile- α motif. Adapted from ^{34, 37, 38}.

Alternative splicing at the 3'end of the p53, p63, and p73 transcripts results in different ΔN and TA isoforms: eight splice variants for p53 (α , β , γ , δ , ϵ , ζ , $\Delta p53$ and $\Delta E6$), five for p63 (α , β , γ , δ and ϵ), and nine for p73 (α , β , γ , δ , ϵ , θ , ζ , η and $\eta 1$) ³⁴.

The full-length p73 isoform (classified as TAp73 α) recognises the same set of p53 transcriptional targets involved in cell cycle arrest (such as p21), apoptosis (such as Bcl-2 and PUMA), among others (Figure 3). It is a critical tumour suppressor that compensates for p53 loss in tumour regression ³⁴⁻³⁶.

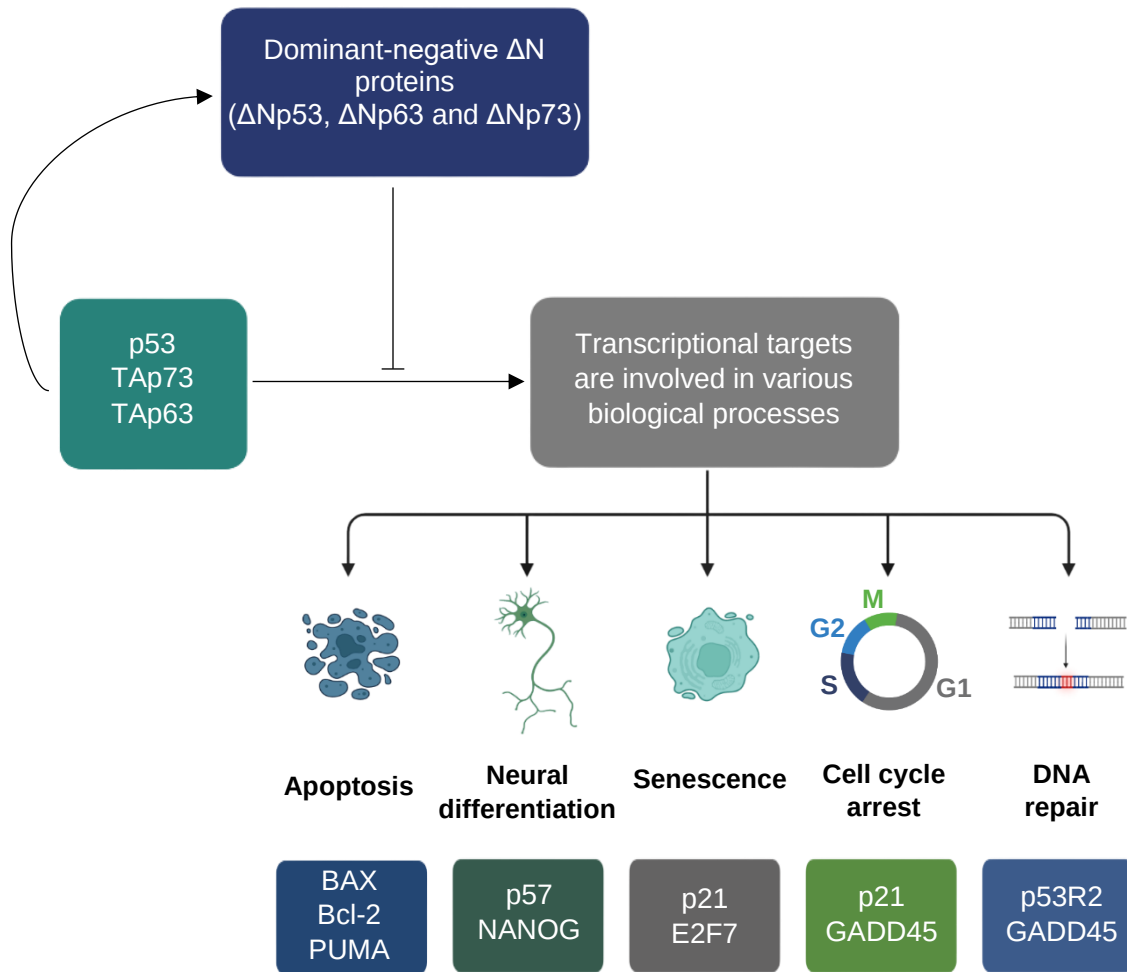


Figure 3. Schematic representation of interactions of p53 family isoforms. ΔNp53, ΔNp63 and ΔNp73 play a dominant-negative role by inhibiting transcriptional and other biological activities of TA isoforms. Adapted from ^{34, 37}.

1.3.1. Role of TAp73 in neuroblastoma

Unlike p53, the *TP73* gene is infrequently mutated in cancer. In the NB, LOH and silencing of TAp73 were observed. The lack of TAp73 expression could be caused by the deletion of one allele at 1p36 or mutations in the TAp73 promoter region ^{39, 40}.

The TAp73 has a major role in NB formation and development since it is associated with neurogenesis and neural differentiation ^{33, 35}. A study revealed that TAp73-deficient mice exhibit neurological, pheromonal and inflammatory defects. However, this is not reflected in the increasing tendency to develop spontaneous tumours ⁴¹.

The contribution of p73 to NB is related to the expression of its isoforms. Briefly, TAp73 and ΔNp73 show opposite roles ^{40, 42}. While TAp73, a tumour suppressor isoform, is involved

in neuronal differentiation, the Δ Np73 isoform appears to be important for neuronal survival, also having oncogenic properties by inhibiting p53 and TAp73^{42,43}. These proteins are inhibited either by the production of heteroduplex (as in the case of TAp73) or by competition for promoter binding (in the case of p53 and TAp73)⁴⁰. Furthermore, high expression levels of Δ Np73 have been associated with unfavourable outcomes in NB likely due to epigenetic modifications⁴².

Another interesting feature in the pathogenesis of NB is the relationship between *MYCN* and TAp73. It appears that TAp73 overexpression downregulates *MYCN* and induces NB cell differentiation^{40,44}. On the contrary, when *MYCN* is overexpressed, TAp73 is downregulated and this correlation was observed both in NB tumours and cell lines⁴⁵. However, as for *MYCC* and its interaction with p73, studies are still contradictory⁴⁰.

1.4. Modulators of p53 family proteins

1.4.1. MDM2 and MDMX

To maintain proper tissue homeostasis, the cellular processes involving the p53 family are strictly regulated^{46,47}. Although the ability of p53, p63, and p73 to respond to cellular stress is required to prevent tumour growth, their cellular levels can be deregulated, resulting in cell death-dependent effects. Excessive cell death, for instance, can damage tissue structure and function, which is a hallmark of ageing, whereas insufficient cell death can result in neoplastic illnesses⁴⁷.

The two major negative modulators of p53 family proteins are the homologs murine double minute 2 (MDM2, also known as HMD2 in humans) and murine double minute X (MDMX, also known as MDM4 and HDM4 for its human analogue). MDM2 and MDMX inhibit the activity of p53 family members in normal cells. In contrast, in response to cellular stresses, an inhibition of MDM proteins allows p53, p63, and p73 to respond to the damage^{39,48-51}. As inhibitors of the p53 family activities, MDM2 and MDMX are carcinogenic when overexpressed^{48,51,52}.

MDM2 was firstly described in 1987 as one of the several genes amplified on double minute chromosomes from a mouse tumourigenic cell line^{53,54}. Later, the oncogenic potential of the *MDM2* gene was demonstrated, with several studies showing that MDM2 interacts directly with p53, suppressing its transcriptional activity^{48,53,54}. MDMX/MDM4 was subsequently identified, through a screening of a mouse complementary DNA (cDNA)

expression library, as a novel protein that shared a structural homology with MDM2, capable of interacting with p53 ^{53, 54}.

MDM2 and *MDMX* encode structurally related proteins of 491 and 490 amino acids, respectively ^{53, 55}. Both proteins present an N-terminal p53 DNA binding domain (p53 DBD), a central acidic domain (AD), a zinc-finger domain (ZFD) and a C-terminal Ring-finger domain (RFD) ^{53, 55, 56} (Figure 4). The major difference between MDM proteins is that only MDM2 possesses a nuclear localization signal (NLS) and a nuclear export signal (NES). These two extra sequences are responsible for the cytoplasmic-nuclear translocations of MDM2, while its absence in MDMX is responsible for its cytoplasmic localization ^{53, 56}. The RFD is required for homo-dimerization and heterodimerization with MDMX via RING-RING interaction in MDM2. Along with the ZFD and AD, the RFD is essential for the MDM2 function as an E3 ubiquitin ligase ⁵³.

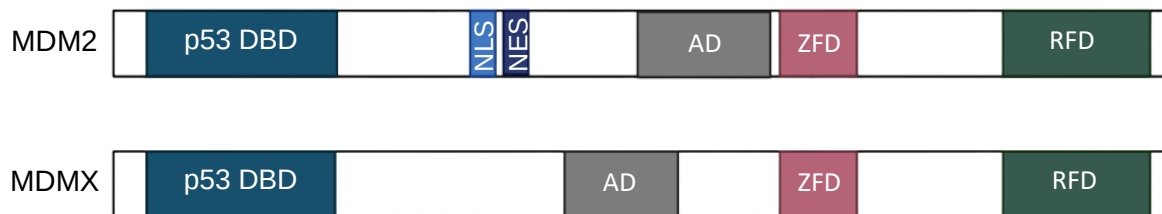


Figure 4. Schematic representation of MDM2 and MDMX domain structures. p53 DBD: p53 DNA binding domain; NLS: nuclear localization signal; NES: nuclear export signal; AD: acidic domain; ZFD: zinc finger domain; RFD: Ring-finger domain. Adapted from ⁵⁵.

1.4.2. p53 and TAp73 interaction with MDM2 and MDMX

According to several studies, MDM2 and MDMX have distinct but complementary roles in p53 regulation. Although both proteins suppress p53 transcriptional activity, they have significant differences in their p53 regulation mechanisms, while MDM2 primarily regulates p53 stability, MDMX controls its activity ⁵³.

As previously mentioned, only MDM2 is capable of acting as an E3 ubiquitin ligase, leading p53 and itself to degradation by ubiquitination, whereas MDMX controls p53 levels by modulating MDM2 levels ⁵³. In cellular homeostasis, MDM2/MDMX and p53 can regulate each other and form a negative feedback loop ^{53, 54}.

MDM2 leads to repression of p53 expression by three different mechanisms ⁵³ (Figure 5):

1. MDM2 binds to the p53 TAD, thereby blocking its transcriptional activity;
2. MDM2 promotes p53 translocation from the nucleus to the cytoplasm through its NES domain, with suppression of p53 transcriptional activity;
3. MDM2 acts as an E3 ubiquitin ligase, that promotes proteasomal-mediated degradation by the ubiquitination of p53.

The p53 ubiquitination by MDM2 can occur in two ways, depending on MDM2 expression levels. Under low levels, MDM2 acts as a homotetramer, inducing monoubiquitination of p53, which promotes the export of p53 from the nucleus to the cytoplasm for proteasomal ubiquitination ^{57, 58}. Instead, high levels of MDM2 form a heterotetramer with MDMX that favours p53 polyubiquitination and finally, p53 nuclear degradation ^{53, 58}.

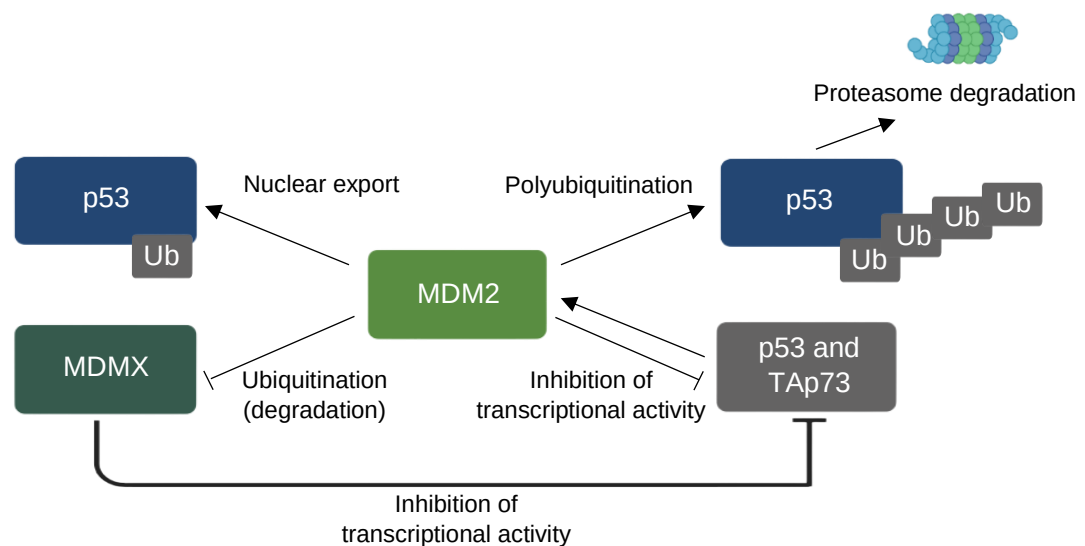


Figure 5. MDM2/MDMX regulation of p53 and TAp73. Ub: ubiquitin. Adapted from ⁵³.

Like MDM2, MDMX can also bind to the p53 TAD, inhibiting its transcriptional activity. MDMX lacks E3 ligase activity and cannot directly promote p53 degradation. Despite this, MDMX can interact with MDM2 via its RING-RING domains, thereby facilitating the nuclear degradation of p53. So, MDMX acts cooperatively with MDM2, potentiating its ubiquitin ligase activity ^{49, 51, 53} (Figure 5).

Given the structural similarity between p53 and p73 (with homologous TAD), it is plausible that MDM proteins may also regulate p73 activity as described for p53 (Figure 5).

Indeed, the binding ability of MDM2 and MDMX to p73 has been well documented ^{39, 48-50}. In fact, MDM proteins can bind TAD of p73 and inhibit its transcriptional activity (Figure 5). However, unlike with p53, MDM2 does not promote ubiquitination of p73 and consequently its degradation ^{39, 49, 50}. Although most literature reports that MDM2 does not promote p73 ubiquitination, a study demonstrated that MDM2, through the use of K11, k29 and k63-linked chains, mediates p73 ubiquitination *in vivo* and *in vitro*. Furthermore, overexpression of MDM2 promotes degradation of p73 mainly through E3 ubiquitin-protein ligase Itch in MDM2-null mouse embryo fibroblasts ⁵⁹. Another study revealed that MDM2 indirectly promoted proteasomal degradation of p73 through functional interaction with Itch in HeLa cells ⁶⁰. Since p73 was only found in a limited subset of cells or tissues, it is plausible that MDM2 regulates it in a cell-specific manner ⁴⁸.

As previously suggested in some studies, the affinity of both MDM2 and MDMX for p73 is on the same order of magnitude as those for p53, but a study showed that p73 interact more strongly with MDMX, suggesting that MDMX may have a stronger impact than MDM2 on the p73 regulation ⁴⁶.

The MDM2 is overexpressed in a wide range of human cancers, including NB, and contributes to the poor response to treatment and poor prognosis in patients with high-risk NB ^{61, 62}. MDM2 expression and activity are typically deregulated in NB and are thought to be, at least in part, responsible for the cytoplasmic sequestration of p53 (an alternative mechanism of inactivation) ³⁵.

An important regulator of the p53-MDM2 network is the tumour suppressor p14^{ARF} (Figure 6). p14^{ARF} inhibits MDM2-dependent p53 degradation, through MDM2-p14^{ARF} complex formation, so maintaining p53 in an active state. As a result, there are multiple mechanisms of p53 inactivation in NB including mutations developed after prolonged pharmacological therapy, overexpression of MDM2 and deletion or downregulation of the *INK4a-ARF* gene encoding p14^{ARF} ^{35, 63}. In fact, the loss of p14^{ARF} has been observed in NB cell lines at relapse ³⁵.

Since MDM2 is a transcriptional target of N-MYC, so the oncogene *MYCN* can also induce p14^{ARF} thereby activating p53 ^{35, 63}. Lastly, p53 can also induce transcription-dependent apoptosis by inducing genes such as *BAX* ⁶³ (Figure 6).

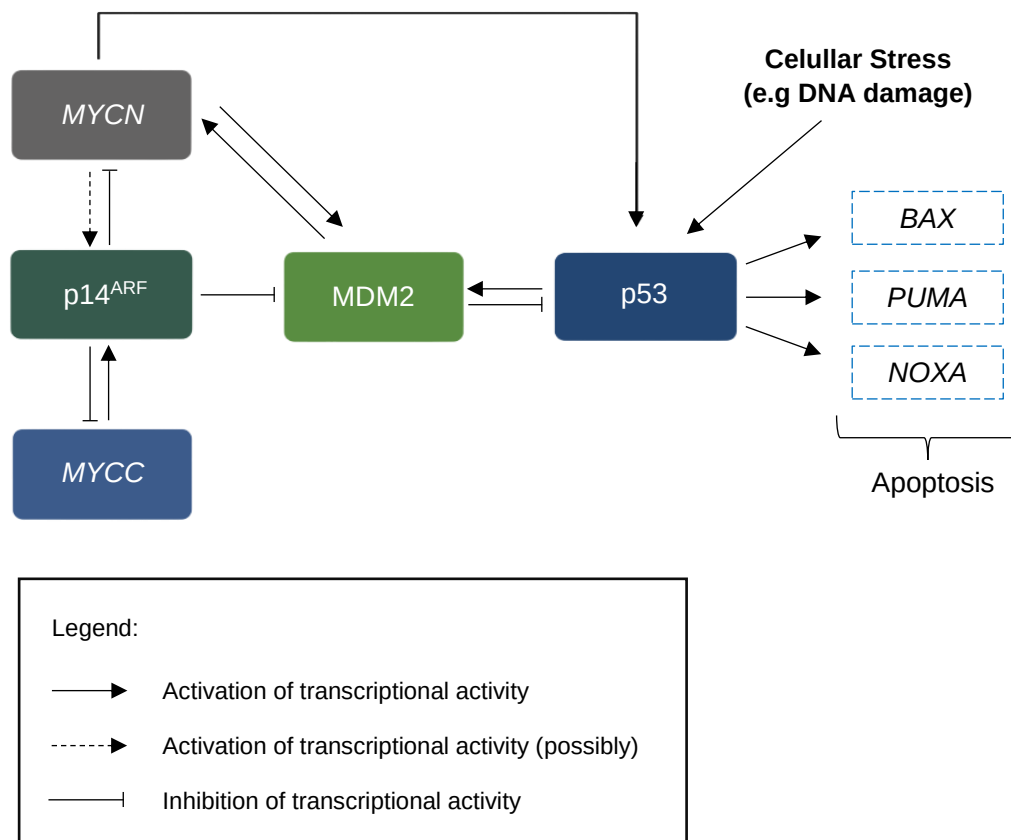


Figure 6. A simplified diagram of the p53/MDM2/p14^{ARF} pathway. In cellular homeostasis, p53 expression is low, primarily due to MDM2. However, in situations of cellular stress induced by internal or external stimuli, which can cause DNA damage, the transcriptional activity of p53 is activated. Subsequently, several cellular responses can occur, such as induction of apoptosis, since p53 can mediate the expression of genes involved with this process (such as *BAX*, *NOXA* and *PUMA*). Furthermore, MDM2 is a direct transcriptional target of p53, induced in response to p53 activation and negatively regulated by p14^{ARF}. The p14^{ARF} inhibits MDM2-dependent p53 degradation and promotes p53 activity. Thus, an autoregulatory feedback loop formed by p53, MDM2, and p14^{ARF} tightly controls p53 expression and activity. In response to abnormal factors including *MYCC* and possibly *MYCN* (dashed line), p14^{ARF} can be activated and conversely p14^{ARF} can suppress the activity of *MYCC* and *MYCN*. *MYCN* directly targets p53 and MDM2. MDM2 can regulate *MYCN* and forms a positive feedback loop. Adapted from ^{63, 64}.

An increasing number of inhibitors of the p53-MDM2 interaction have been proposed, such as nutlins, benzodiazepines, spiro-oxindoles, xanthenes, among others ^{53, 65-67}. MDM2 inhibitors bind to a hydrophobic pocket in the MDM2 protein that interacts with p53, and they have proven effective in preclinical studies, particularly against tumours with at least one copy of *wt*p53 ⁶⁵. In fact, this strategy has been widely followed in the development of clinical trials because MDM2 antagonists are safe for normal tissues and activate the tumour suppressor function of *wt*p53 ⁶⁵.

The first potent and selective antagonists of MDM2 were the small molecule nutlin, namely Nutlin-3. This compound competitively blocks the p53-binding site of MDM2 by binding in the p53-binding pocket within MDM2 with a high degree of specificity, and therefore prevents the tagging of p53 for proteasome-mediated degradation. Thus, it leads to the stabilisation of p53 and activation of the p53 pathway^{35, 65, 66, 68}.

Nutlin-3 is considered cytotoxic in wtp53-expressing cancer cells. Thus, it is essential to consider the *TP53* mutational status⁶⁶. Nutlin-3 is much less effective at inhibiting MDMX-p53 interaction versus MDM2-p53⁵¹. Despite this, due to its suboptimal bioavailability, it was never taken beyond the preclinical stage⁶⁵.

Another MDM2 inhibitor, Idasanutlin (RG7388), has been discovered. Clinical treatment with Idasanutlin in patients with acute myeloid leukaemia showed a durable response, including a complete response superior to 12 months, so these results encouraged further clinical trials. This compound is currently in Phase I/II clinical trials, notably for NB, as a monotherapy or in combination with other drugs, including venetoclax and chemotherapy agents⁶⁵.

The small-molecule RITA (reactivation of p53 and induction of tumour cell apoptosis) also disrupts the p53/MDM2 and MDMX complex, which allows p53 activation and apoptosis induction, in NB cells. Unlike other known inhibitors, RITA achieves this by binding to p53 instead of MDM2. Furthermore, in NB cells, RITA can reactivate mutant p53 (mutp53), and this may be related to the fact that it interferes with the conformation of p53 and not MDM2^{66, 69}.

One study evaluated the ability of Nutlin-3 to sensitize a p53-null and doxorubicin-resistant NB cell line, LA155N, to doxorubicin³⁵. The study concluded that after Nutlin-3 treatment, TAp73 is released from MDM2 and activated by doxorubicin to induce apoptosis. This study addressed the p53-independent mechanisms of Nutlin-3 in chemoresistant NB cells, showing strong clinical relevance for chemoresistant and dysfunctional p53 NB³⁵.

1.4.3. p53 and TAp73 interaction with mutant p53

TP53 is one of the most commonly mutated genes in human tumours and mutations can occur in both germline and sporadic contexts. In contrast to germline mutations, the presence of *TP53* mutations in sporadic cancers appears to correlate with exposure to environmental factors (carcinogens such as ultraviolet radiation and tobacco smoke)⁷⁰.

Alterations in *TP53* include missense mutations, frameshift deletions and insertions, nonsense mutations, silent mutations and other infrequent alterations⁷⁰.

Although there are various types of mutations in the *TP53* gene, missense mutations, which are defined by the substitution of a single amino acid in a codon, are the most frequent in human neoplasia. Missense mutations result in the loss or diminution of the wtp53 activity, especially its tumour suppressor function. These mutations are mainly found in the p53 DBD (over 80%)^{70 71}, indicating that DNA binding is important for tumour suppression, particularly in six “hotspots” codons: 175, 245, 248, 249, 273 and 282. These codons code for residues that are crucial to the structure and function of p53⁷¹. In addition, missense mutations can be classified into two main groups: contact and conformational mutations. Contact mutations directly affect amino acid residues that come into contact with DNA, and thus the DNA-binding ability of the mutp53, e.g. R248Q, R248W, R273H, R273C and R280K. Conformational mutations change the folding and structure of p53. In other words, disrupt the three-dimensional structure of the protein and indirectly affects the DNA-binding ability of the mutant protein, e.g. G245S, G245D, R249S, R175H, Y220C and R282W⁷¹.

Furthermore, p53 missense mutations are closely related to cancer development and more aggressive tumours with poor prognosis. These findings led to the concept that missense mutations are preferentially selected in cancers because they have some specific pro-oncogenic GOF^{70, 72}. These GOF activities drive tumour growth through stimulation of several hallmarks of cancer, including invasion, cell proliferation, genomic instability, migration, angiogenesis, cell death resistance, among others⁷³.

In addition, many p53 mutants have a dominant-negative (DN) effect on wtp53, namely occurring when a missense mutation occurs in one allele of p53, which can inactivate the coexpressed wt allele. Several *in vivo* studies showed that mutp53 is capable of inhibiting wtp53 activity by forming heterotetramers with mutp53 or by inhibiting the binding of wtp53 to its target gene promoters^{70, 74}. In particular, the DN effect only occurs when tumour cells have high levels of mutp53 expression, which allows to conclude that this effect is important in treatment because these high levels of protein are linked to radiation therapy and chemotherapy resistance^{70, 74}. Therefore, it is critical to discover new strategies to abolish the DN effect of mutp53 on wtp53 for an effective treatment.

In fact, p53 mutants are molecules highly involved in chemoresistance to cancer cells, namely by regulating the expression of genes such as multidrug resistance 1 (*MDR1*), which confer drug resistance to mutp53-expressing tumour cells⁷⁵.

In NB, p53 mutations at diagnosis are uncommon, occurring in <2% of cases. In fact, most of the reported mutp53 are associated with relapsed cases and poor outcomes ⁷⁶. In particular, it was investigated the frequency of aberrations of the p53/MDM2/p14^{ARF} pathway in various NB cell lines and reported that most cells established at relapse have alterations of the p53 pathway and in some cases, this is associated with chemoresistance ⁷⁶. Hence, an important mechanism of intrinsic chemoresistance in tumour cells is an abnormality in the p53/MDM2/p14^{ARF} pathway ⁷⁶ (Figure 6).

In addition to inactivating the p53 pathway in NB, overexpression of *MDR-1* has also been shown to play a role in drug resistance in specific subgroups of primary NB ⁷⁶.

Besides the interaction between mutp53 and p53, mutp53 may inhibit the transcriptional activity of other p53 family members, including p63 and p73, blocking their capacity to suppress growth and induce apoptosis ^{50, 77}. Due to these GOF characteristics, the mutp53 can be considered an obstacle in the treatment of cancer.

There are several strategies to target mutp53, including promotion of the degradation of mutp53 through proteasome and autophagy pathways, interference with the interaction between mutp53 and other proteins, restoration of wtp53 activity to mutp53, and interference in signalling pathways downstream of mutp53 ^{75, 77} (Figure 7).

Regarding the therapeutic strategy that promotes the degradation of the mutp53, it is known that both wt and mutp53 can be targeted for proteasomal degradation in normal cells by MDM2. Besides MDM2, another chaperone-associated E3 ubiquitin ligase, carboxyl terminus of Hsc70-interacting protein (CHIP), has been found to be critical for the degradation of mutp53 ^{75, 77}. Several indirect strategies have been proposed to destabilize mutp53, including heat shock protein 90 (HSP90) inhibitors, histone deacetylase (HDAC) inhibitors or *SIRT1* activators, which trigger the degradation of mutp53 by E3 ligases MDM2 or CHIP ^{73, 75, 77}. Degradation of mutp53 can also occur by autophagy, namely macroautophagy, which is the process by which intracellular content is engulfed and degraded via lysosomes. The degradation of mutp53 has increased specifically by glucose restriction-induced macroautophagy ^{75, 77} (Figure 7A).

Regarding the interaction of mutp53 with other proteins (Figure 7B), specifically TAp73, a small molecule known as RETRA was identified to destabilize the mutp53 and TAp73 interaction ⁷⁸. Treatment of cancer cells that express mutp53 with RETRA causes a significant

increase in the expression level of TAp73 and therefore an activation in TAp73 transcriptional activity^{77, 78}.

Another therapeutic alternative is the reactivation of mutp53, converting it into a wt-like tumour suppressor (Figure 7C). A variety of compounds capable of restoring wtp53 function have been identified, including P53R3, NSC319726, PK7088, MANIO, PEITC, RITA, COTI-2, PRIMA-1 and APR-246/PRIMA-1^{MET}. APR-246, also known as PRIMA-1^{MET}, is a more potent form of PRIMA-1 for reactivating mutp53 and inducing apoptosis^{73, 75, 77, 79}.

Rather than targeting mutp53 directly, other targets for therapeutic intervention are downstream pathways regulated by mutp53 (Figure 7D). Mutp53 can elevate the expression of mevalonate pathway genes and this pathway is involved in several aspects of tumourigenesis, such as proliferation, survival and invasion. Thus, inhibition of the mevalonate pathway may be a therapeutic strategy for tumours carrying p53 mutations. In particular, statins (drugs used to reduce cholesterol levels) by causing the inhibition of cholesterol synthesis decreased the survival of mutp53 cells, being also used as anticancer agents, although their antitumorigenic mechanism is still not completely clear^{73, 77, 80}.

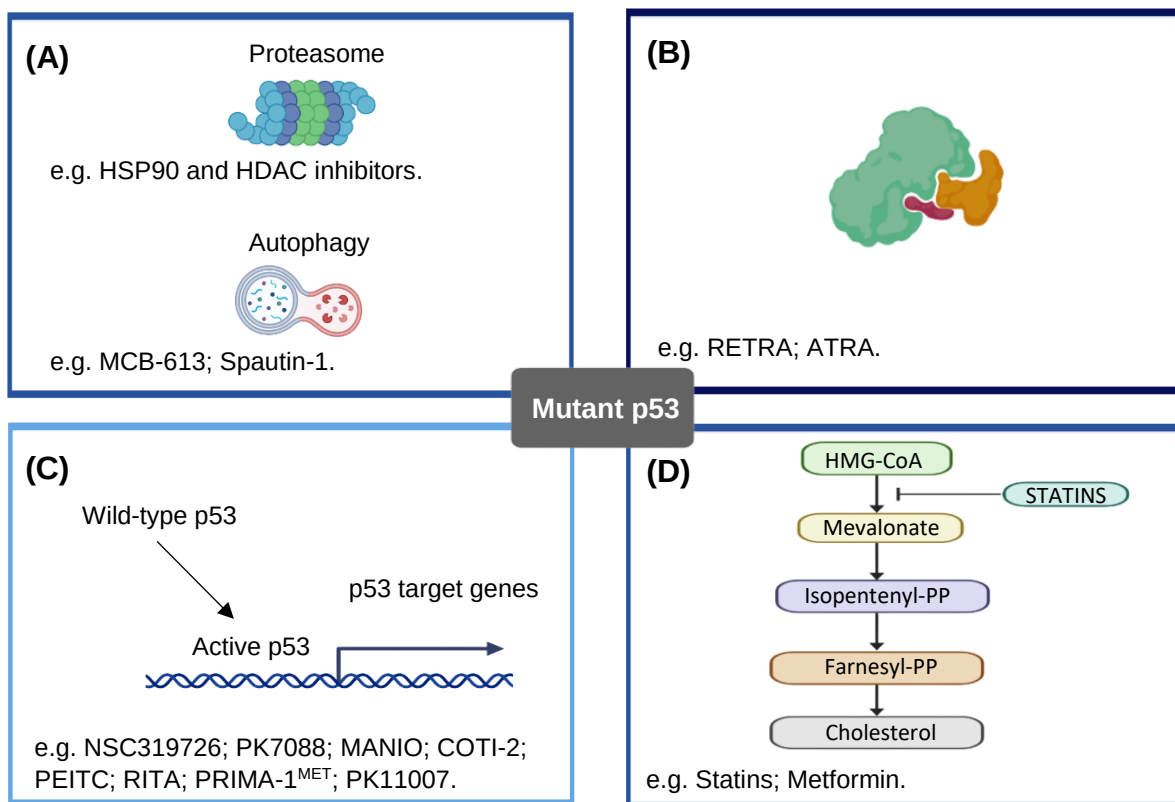


Figure 7. Strategies of targeting mutant p53. (A) Degradation. **(B)** Inhibition of the interaction with other proteins. **(C)** Reactivation. **(D)** Inhibition of downstream signalling pathways. Adapted from^{75, 77, 79, 81}.

1.5. MicroRNAs

MicroRNAs (miRNAs) are a class of small single-stranded non-coding RNAs with about 20-22 nucleotides (nt) in length^{82, 83}. These transcripts are located within the introns or exons of protein-coding genes (30%) or in intergenic areas (70%)^{84, 85}. It is estimated that 30 to 60% of human genes are regulated by miRNAs⁸⁴. The founding members of the class of miRNAs are the products of the *lin-4* and *let-7* genes of *Caenorhabditis elegans*, discovered in 1993 and 2000, respectively⁸². Since their discovery, thousands of miRNA sequences have been described in various organisms, including animals, plants, and viruses^{82, 84, 86}. Many miRNAs are conserved throughout evolution and play important roles in regulating gene expression at the post-transcriptional level by targeting the 5' untranslated region (UTR), coding regions or 3'UTR of mRNA. By binding to complementary regions of the mRNA through base pairing, they are able to inhibit translation or cause degradation of the mRNA^{82, 87}.

Interestingly, miRNAs play crucial roles in a wide variety of biological processes, such as cell proliferation, differentiation, apoptosis, stress response, adhesion, migration, and invasion. Since miRNAs maintain cell homeostasis, their dysregulation is associated with diseases, including autoimmune, cardiovascular, muscle-skeleton and oncologic diseases^{84, 86}. Importantly, a single miRNA can bind to and regulate many different mRNA targets, and conversely, several different miRNAs can cooperatively bind to and control a single mRNA target. As such, miRNAs have been considered promising therapeutic targets⁸⁴.

1.5.1. The biogenesis and nomenclature of miRNAs

The biogenesis of miRNAs in mammals is tightly controlled at several steps, including transcription in the cell nucleus, export to the cytoplasm, and subsequent processing and maturation⁸⁸. First, miRNA genes are transcribed by the RNA polymerase II enzyme into primary/primitive miRNA transcript (pri-miRNA), which are 5'- capped and 3'- polyadenylated^{85, 89}.

The pri-miRNA acquires hairpin-like secondary structures, such as "stem" regions, in which two RNA segments with complementary bases are paired, and "loop" regions, in which the base pairs are not complementary, thus constituting circular loops. Inside the nucleus, the pri-miRNAs are cleaved by the microprocessor, a multiprotein complex containing Drosha (nuclear ribonuclease (RNase) III enzyme) and its partner protein/cofactor, DGCR8 (DiGeorge Syndrome Critical Region 8), into shorter structures, called precursor miRNA (pre-miRNA,

approximately 70 nt in size). The pre-miRNA is exported to the cytoplasm via the GTP-binding nuclear protein Ran (RanGTP)/exportin-5 (XPO5) complex ^{85, 89} (Figure 8).

In the cytoplasm, the pre-miRNAs are processed by Dicer (RNase III enzyme), which removes the loop in the stem-loop structure, resulting in the formation of a mature miRNA duplex. This duplex is incorporated into the RNA-induced silencing complex (RISC), in which the two RNA strands are separated ⁸⁹. One of these strands remains associated with the RISC, constituting the mature miRNA (with size between 19 and 24 nt), and binds specific mRNAs targets according to sequence complementarity, while the complementary strand undergoes degradation ^{85, 88} (Figure 8). Nonetheless, recent evidence also shows that miRNA biogenesis can be microprocessor-independent. Examples include pre-miRNA-like hairpins called "Mirtrons" formed from spliced and disbranched short hairpin introns, some small nucleolar RNAs (snoRNAs) and endogenous short hairpin RNAs (shRNAs) ⁸⁵.

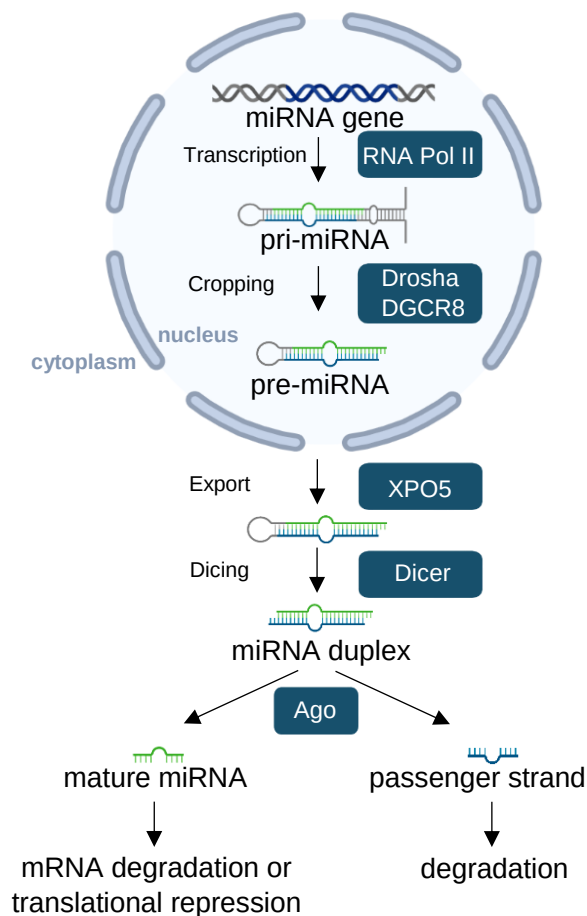


Figure 8. Canonical miRNA biogenesis pathway. In the nucleus, the microRNA gene is transcribed by RNA polymerase II (RNA Pol II) to generate the primary miRNA transcript (pri-miRNA). The pri-miRNA is cleaved by the Drosha-DGCR8 complex, which includes the ribonuclease (RNase) type III enzyme called Drosha and the RNA binding protein DGCR8. The precursor miRNA (pre-miRNA) is subsequently generated and exported to the cytoplasm by exportin 5 (XPO5). In the cytoplasm, the pre-miRNA is processed by the dicer (a cytoplasmic RNase III) and its partners to form a mature miRNA duplex. The miRNA duplex is incorporated into Argonaute (Ago) protein with RNA-induced silencing complex (RISC). Then, the miRNA duplex is separated by helicase, and one strand is normally chosen as the mature miRNA, whereas the “passenger” strand is degraded. The mature miRNA can then bind to the target messenger RNA (mRNA) and exert its inhibitory function through translational repression (in case of multiple sequence mismatches between the target mRNA and the miRNA) or degradation of the mRNA (in case of perfect base complementarity). Adapted from ⁹⁰⁻⁹².

After the discovery of thousands of miRNAs, it became necessary to find criteria to name them appropriately. Thus, several miRNA sequence databases emerged, being miRBase the most commonly used reference database by the scientific community ⁸⁸.

Typically, the numbering of miRNAs follows a sequential order. The prefix “mir-” refers to pri-miRNA and pre-miRNA, while the prefix “miR-” (with a capitalized R) is used for mature miRNA. Both prefixes are followed by a hyphen and a unique identifying number ⁸⁵.

The main features of miRNA nomenclature are as follows ^{85, 86}:

- The species of origin is designated with a three or four letters prefix, for example, “hsa-” (*Homo sapiens*) in the case of a human miRNA, followed by a numeric suffix.
- Other numeric suffixes are used to designate pre-miRNAs, pri-miRNAs, and genes leading to mature miRNAs that are 100% identical, but located at various sites in the genome.
- miRNAs from the same species with similar sequences that differ by 1 or 2 nt, are identified by suffixes with an additional lowercase letter.
- The suffix “-3p/-5p” is added to the names of two mature miRNAs when they originate from opposite arms of the same pre-miRNA and are present in similar quantities.

Genes encoding miRNAs from other organisms can also be named using the three-letter prefix, with capitalization, hyphenation, and/or italics, according to species-specific genetic nomenclature conventions ⁸³.

1.5.2. miRNAs and cancer

As previously mentioned, miRNAs play important roles in biological pathways in various organisms. The expression profiles of miRNAs are significantly altered in various types of cancer when compared to corresponding normal tissues, and differences can also be observed between different subtypes within the same cancer type or between patients who have the same cancer type but different prognoses. Aberrant miRNA expression can affect a multitude of transcripts and influence cancer-related signalling pathways. Thus, miRNAs can regulate several cancer-relevant processes, such as proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism^{84, 93}.

The main causes of altered miRNA expression in cancer include genetic alterations, epigenetic mechanisms, regulation of miRNAs by transcription factors in cancer (miRNA suppression by oncogenic transcription factors; negative miRNA regulation by loss of tumour suppressor transcription factors) and mutations in miRNA biogenesis pathways^{93, 94}.

The miRNAs can act as oncogenes (referred to as "oncomiRNAs") or tumour suppressor miRNAs ("oncosuppressor miRNAs" or "TSmiRs"), depending on the functions of the target protein(s) they regulate (Figure 9). OncomiRNAs are overexpressed in malignant tumours, act by stimulating cell proliferation and inhibit the action of tumour suppressor genes and genes that control apoptosis. While tumour suppressor miRNAs are downregulated in malignant tumours and act against the development of neoplasms by inhibiting oncogenes. Furthermore, an individual miRNA can also potentially perform dual functions, being both oncogene and tumour suppressor if its targets include growth-promoting genes and antiproliferative genes, respectively^{84, 85, 93}.

In summary, miRNAs can be considered promising molecular biomarkers, as they have the potential to be used as tools or targets for new therapies of different types of cancer, to monitor treatment response, and to make adjustments to therapeutic intervention. In addition, they have several advantageous, including small size, stable molecules resistant to various adverse conditions (such as temperature or pH changes), easy to quantify, presence in various body fluids (blood, urine, saliva, serum, plasma, and others fluids), and encapsulation in vesicles or protection by proteins (exosomes and microvesicles, protein complexes, high-density lipoproteins)^{84, 85}.

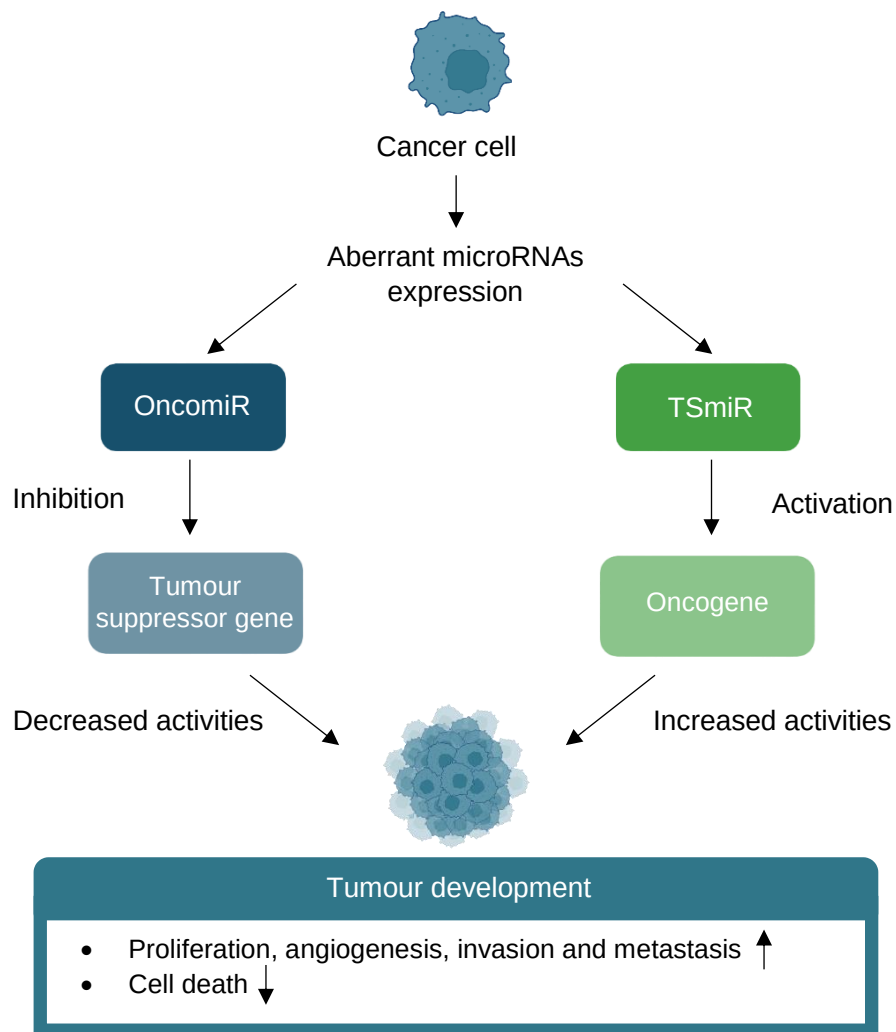


Figure 9. miRNAs as oncogenes or tumour suppressor genes. The miRNAs influence cancer molecular pathways by targeting oncogenes and tumour suppressors. In malignant cells, increased expression of oncomiRs inhibits tumour suppressor genes. Decreased expression of TSmiRs potentially enhances the expression of oncogenes. As a result, both oncogenic and tumour suppressor miRNAs lead to tumour formation. OncomiR: oncogenic miRNAs; TSmiR: tumour suppressor miRNAs; ↑ : induces/increases; ↓ : inhibits/reduces. Adapted from 84, 91, 95.

1.5.2.1. miRNAs as therapeutic tools in neuroblastoma

Since NB is more frequent in children and given the available treatment options (chemotherapy, radiotherapy and surgery) as well as the complications that can arise from them such as severe side effects from chemotherapeutic drugs, the search for alternative therapeutic strategies that are more appropriate to their age group is critical ^{8, 15}. Therefore, miRNAs emerge as an alternative tool.

Several studies have been conducted to better understand the role of miRNAs in the treatment of NB. In particular, it has been discovered that they can help to understand the pathophysiology of NB, since particular miRNAs affect essential processes, such as apoptosis and differentiation ⁸. As a result, some miRNAs have already been described for their role in disease progression (Table 2) and inhibition (Table 3) of NB, and which molecular targets are affected.

The use of miRNA-based drugs targeting restoration of expression of tumour-suppressor miRNAs or inhibition of miRNAs acting as oncogenes, could be a treatment for NB ¹⁵. In fact, a study from Tivnan and co-workers showed that the first targeted delivery using nanoparticles conjugated with GD₂ antibody and a tumour suppressor miRNA, miR-34a, caused a considerable reduction of NB tumour growth ¹⁰⁴. In NB, the use of miRNAs is yet in the preclinical phase, so further research is needed to better understand the biological function of miRNAs in NB, as well as the delivery approach of miRNA-mediated therapy in order to improve its safety and validate its applicability in children ^{8, 15}.

Table 2. Selection of relevant OncomiRs in NB.

miRNA	Expression	Target	Function	References
miR-15a	Upregulation	<i>RECK</i>	Induces migration and invasion	⁹⁶
miR-21	Upregulation	<i>PTEN, PDCD4, FOXO3A</i>	Induces proliferation	⁹⁷
miR-23a	Upregulation	<i>CDH1</i>	Induces migration and invasion	⁹⁸
miR-192	Upregulation	<i>Dicer1</i>	Induces proliferation and migration	^{99, 100}
miR-380-5p	Upregulation	p53	Decreases cell death	¹⁰¹
miR-558	Upregulation	HPSE	Induces proliferation, invasion, metastasis and angiogenesis	¹⁰²
miR-1303	Upregulation	<i>GSK3β, SFRP1</i>	Induces proliferation	¹⁰³

Table 3. Selection of relevant TSMiRs in NB.

miRNA	Expression	Target	Function	References
Let-7	Downregulation	<i>MYCN</i>	Induces differentiation	^{105, 106}
miR-9	Downregulation	<i>MMP-14</i>	Inhibits invasion, metastasis and angiogenesis	¹⁰⁷
miR-34a	Downregulation	<i>MYCN, E2F3, BCL2</i>	Induces cell cycle arrest and apoptosis, and reduce angiogenesis	^{108, 109}
miR-203	Downregulation	<i>SAM68</i>	Inhibits invasion, proliferation and migration	¹¹⁰
miR-338-3p	Downregulation	<i>PREX2a</i>	Inhibits proliferation and survival, and induce cell-cycle arrest	¹¹¹
miR-1247	Downregulation	<i>ZNF346</i>	Inhibits proliferation, and induce cell-cycle arrest and cell death	¹¹²

Furthermore, examples of miRNAs and biological processes associated with the tumour suppressor role of TAp73 in NB are provided in Figure 10 ^{100, 113, 114}.

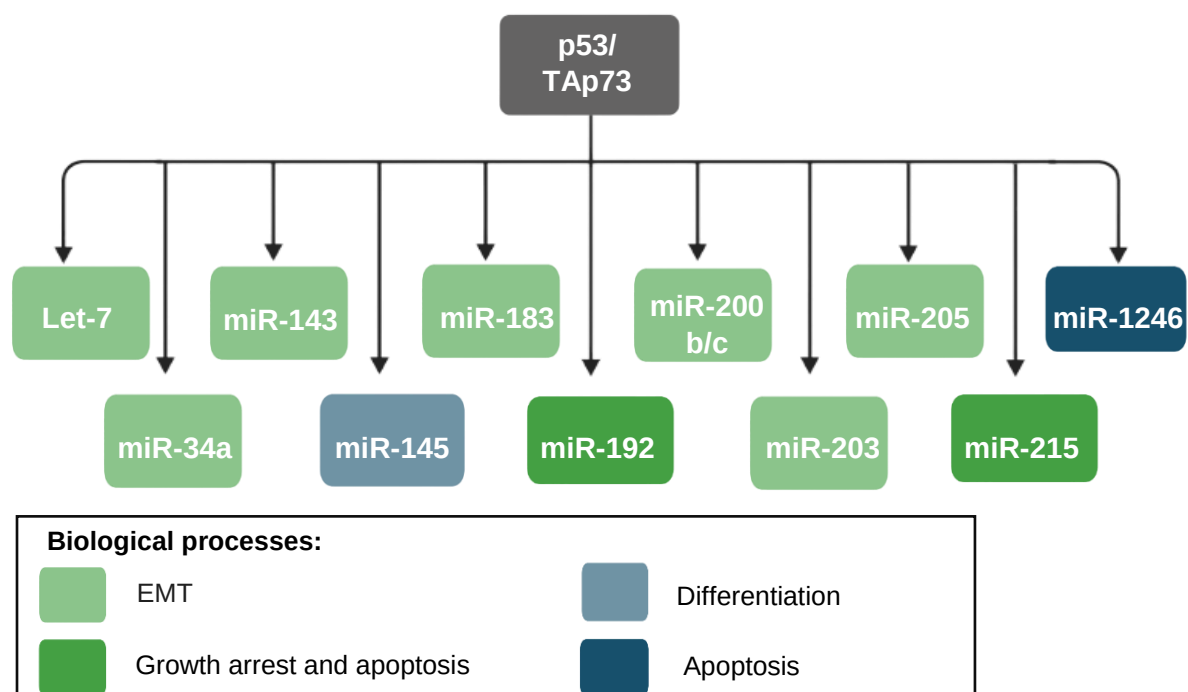


Figure 10. p53/TAp73-miRNAs interactions. miRNAs targeted by p53/TAp73.

1.6. Yeast as a model to study p53 family proteins

Yeast is a powerful eukaryotic model organism that enables a better understanding of human biology and disease. The yeast *Saccharomyces cerevisiae* is one of the most widely used model organisms, which has contributed to the elucidation of proteins and molecular mechanisms underlying several human diseases, such as cancer ¹¹⁵⁻¹¹⁷.

The use of *S. cerevisiae* as a model organism is supported by several advantages, including short generation time, easy and low-cost manipulation, fully sequenced genome, high degree of conservation of cellular and molecular mechanisms with human cells, among others ¹¹⁵⁻¹¹⁷. Since it is a unicellular organism, its main limitations are the impossibility to study aspects of the disease such as multicellularity and cell-cell interactions ¹¹⁷.

Depending on the conservation of the underlying pathways, there are two main approaches for their study in yeast. If the gene implicated in human disease is conserved in yeast, its function can be studied directly. However, if the gene implicated in human disease has no orthologues in yeast, the heterologous expression (also referred to as "humanized yeast"), will allow its study in yeast, without the interference of other similar human proteins ^{116, 117}.

S. cerevisiae has been used to understand the biology and pharmacology of the p53 family. In the case of this family of proteins and their negative regulators (MDM2 and mutp53), no orthologous genes have been identified in this organism, which has allowed the study of the activity of human p53 family proteins by heterologous expression ¹¹⁸.

Several yeast cell-based assays have already been developed for functional and pharmacological studies of p53 family proteins ^{117, 119}. An example is the yeast phenotypic assay, which aims to identify potential compounds capable of inhibiting the interactions between p53 family proteins and their negative regulators, by monitoring the growth of yeast cells ¹¹⁹⁻¹²¹ (Figure 11). The yeast phenotypic assay can be considered an effective screening assay for the discovery of promising compounds targeting p53 family proteins. The molecular mechanism of action of these compounds should then be validated in human tumour cell lines.

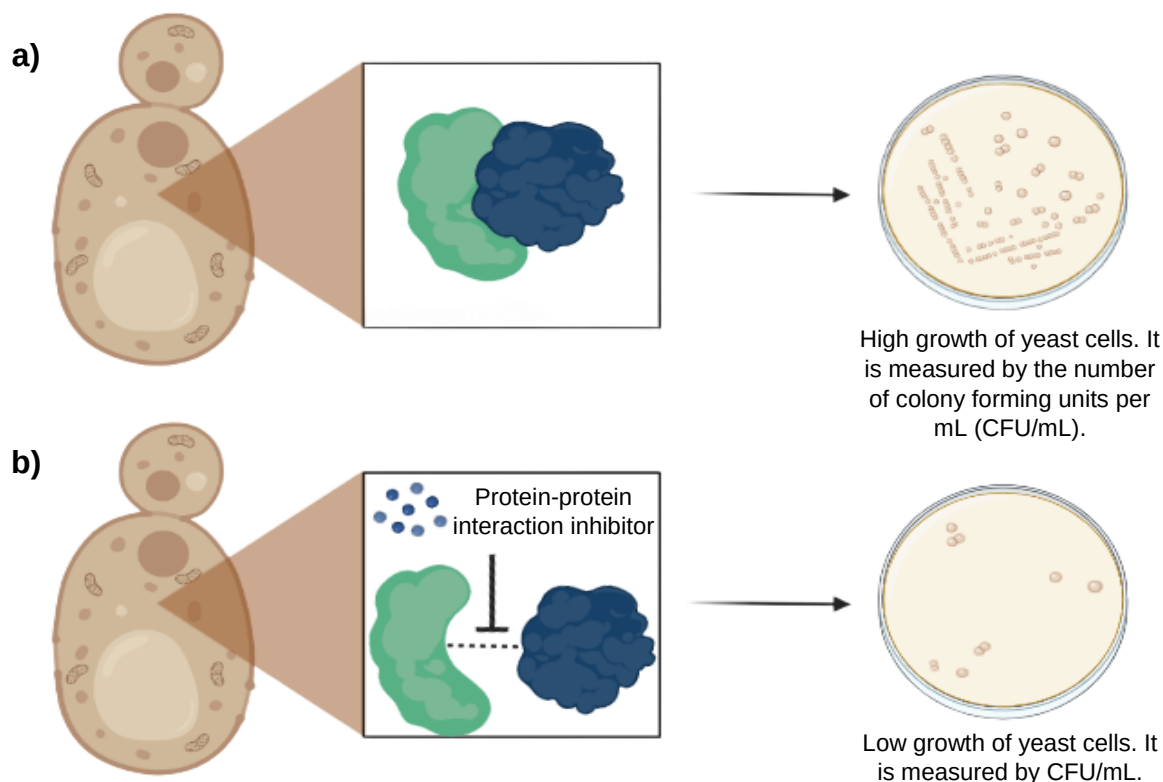


Figure 11. Yeast phenotypic assay. **a)** Yeast cells co-expressing p53 family protein (e.g. TAp73) and negative regulator (such as MDM2 or mutp53). The negative regulator inhibits the p53 family-induced yeast growth inhibition; **b)** The treatment with an inhibitor of protein-protein interaction re-establishes the p53 family-induced yeast growth inhibition. Green protein icon: mutp53/MDM2; blue protein icon: p53 family proteins.

1.7. Xanthenes as anticancer agents, potential p53 family-activating agents

Xanthenes (with an IUPAC name of 9*H*-xanthen-9-one) are heterocyclic planar compounds with a dibenzo- γ -pyrone structure, originally isolated as secondary metabolites from plants and microorganisms. The general structure of the xanthone is shown in Figure 12

¹²².

The xanthone derivatives show several pharmacological properties such as antioxidant, anti-inflammatory, anti-allergic, antidiabetic, antimicrobial, antifungal, antiviral activities and of particular attention, the anticancer activity, which depends on the various structures modified by xanthone ring substituents ¹²²⁻¹²⁴. This wide range of biological activities is due to the fact that xanthone derivatives have the ability to bind to multiple protein receptors ¹²².

Xanthenes have been shown to have potential chemopreventive and chemotherapeutic properties at various stages of carcinogenesis and include cell cycle arrest, suppression of tumour cell proliferation, induction of apoptosis and differentiation, reduction of inflammation,

and inhibition of adhesion, invasion, and metastasis¹²⁴. The anticancer activity depends on the type, number and position of functional groups connected to the xanthone structure¹²².

The simple structure of xanthone derivatives and their diverse pharmacological properties have motivated many researchers to isolate these compounds from natural resources or synthesise them in order to discover new drugs. Several studies have demonstrated the anticancer potential of xanthone derivatives by inhibiting the proliferation of various human cancer cell types, regulating various targets and signalling transduction pathways, through *in vitro* and *in vivo* assays. However, *in vivo* and clinical trials remain limited^{122, 124}.

Antitumour activity associated with p53 family proteins activation has been described in both natural and synthetic xanthenes, being a promising strategy for the treatment of various cancers, including NB.

Concerning natural xanthenes, α -Mangostin and gambogic acid (Figure 12) are examples of prenylated xanthenes that have potent cytotoxic activity against human cancer cells and particularly, α -mangostin induces apoptosis in human mutp53 cancer cells. Furthermore, these xanthenes can increase p53 transcriptional activity and inhibit the p53-MDM2 interaction¹²⁵. A yeast-based screening confirmed the disruption of the p53-MDM2 interaction by these xanthenes and *in silico* studies revealed that the binding interactions of these xanthenes with MDM2 are similar to that of nutlin-3a¹²⁶.

Regarding synthetic xanthenes, a study conducted by Leão and co-workers⁶⁷, led to the identification of a xanthone compound, 3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano(3,2-b)xanten-6-one (pyranoxanthone 1, also known as LEM1) (Figure 12), by virtual screening of a library of xanthone derivatives. LEM1 was shown to be effective against tumour cells with wtp53 and by acting as an inhibitor of the p53-MDM2 interaction. The compound binds to MDM2 in a similar manner to nutlin-3a to MDM2⁶⁷.

In 2019, Gomes and co-workers¹¹⁹ conducted a study in which they identified another xanthone, 3,4-dimethoxy-9-oxo-9H-xanthene-1-carbaldehyde (LEM2) (Figure 12). LEM2 exhibited potent antitumour activity against patient-derived NB cells. This compound inhibits TAp73-MDM2 and TAp73-mutp53 interactions, resulting in TAp73 activation and induction of cell cycle arrest and apoptosis in NB cell lines. Furthermore, LEM2 revealed synergistic effects with chemotherapeutics such as doxorubicin and cisplatin. Despite the promising activity of this xanthone, its low aqueous solubility has limited *in vivo* studies¹¹⁹.

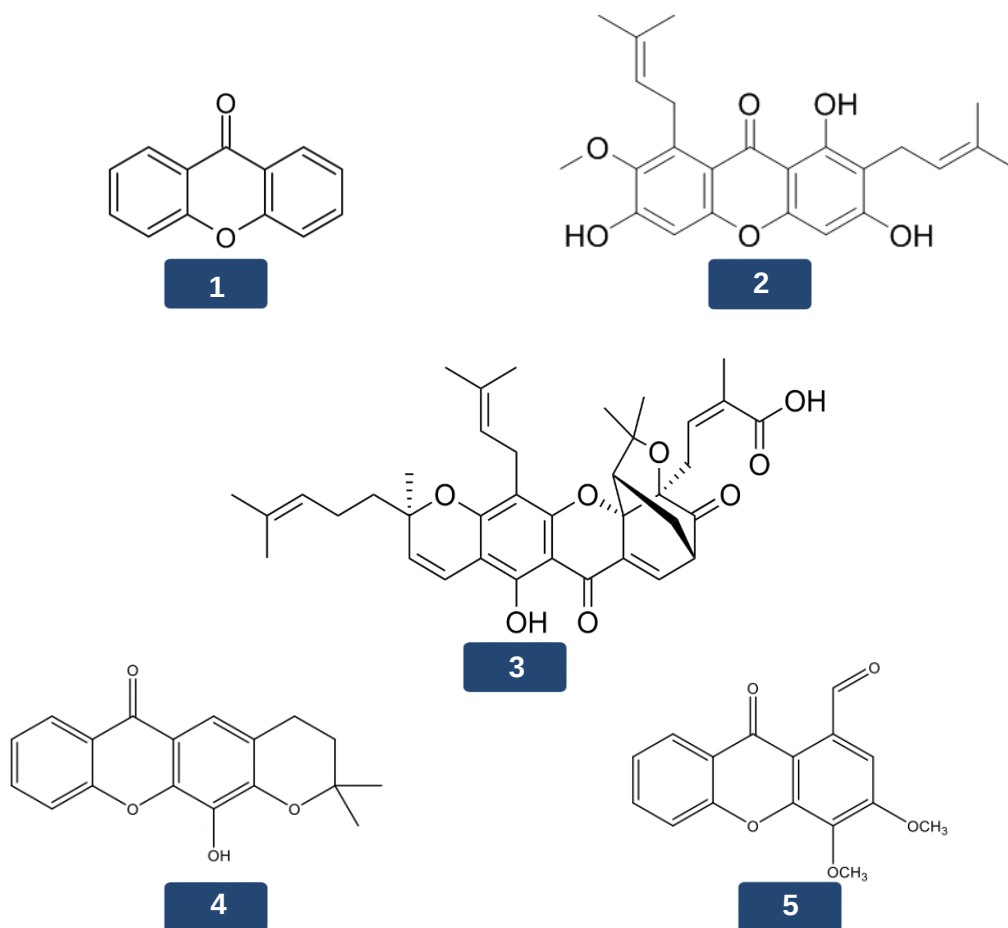


Figure 12. Chemical structures of xanthones. (1) basic structure of xanthone; (2) α-mangostin; (3) gambogic acid; (4) 3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano(3,2-b)xanten-6-one (LEM1); (5) 3,4-dimethoxy-9-oxo-9H-xanthene-1-carbaldehyde (LEM2).

2. AIMS

The p53 family proteins are promising therapeutic targets in the treatment of neuroblastoma. Our previous work ¹¹⁹ demonstrated a promising antitumour activity of the xanthone LEM2 against this type of cancer. Despite this, its low solubility did not allow its *in vivo* study in mouse models. Due to this limitation of LEM2, a small library of LEM2 derivatives was synthesised, and the 1-(dibromomethyl)-3,4,6-trimethoxy-9H-xanthen-9-one (LEM3) was selected based on its improved drug-like properties. Based on these considerations, this work aimed to better understand the pharmacology of p53 family proteins (specifically TAp73). To achieve this goal, it was intended to validate:

1. The molecular mechanism of action of LEM3 as a TAp73-activating agent;
2. The antitumour activity of LEM3 in NB cells, in particular, its interference with non-coding RNAs, namely, miRNAs.

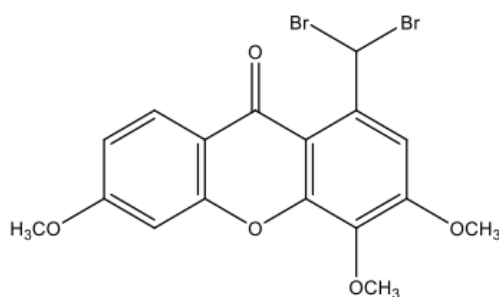


Figure 13. Chemical structure of 1-(dibromomethyl)-3,4,6-trimethoxy-9H-xanthen-9-one (LEM3).

3. MATERIALS AND METHODS

3.1. Compound

The LEM2 analogue, 1-(dibromomethyl)-3,4,6-trimethoxy-9H-xanthen-9-one (LEM3) was kindly provided by Prof Emília Sousa from the Faculty of Pharmacy, University of Porto. LEM3 was dissolved in dimethyl sulfoxide (DMSO) from Merck Millipore (Taper, Sintra, Portugal).

3.2. Human cell lines and growth conditions

The human cell lines used are described in Table 4. The human neuroblastoma SH-SY5Y cell line expressing wtp53, the human neuroblastoma SK-N-BE(2) cell line with mutp53, and a non-tumorigenic foreskin fibroblasts HFF-1 cell line were purchased from ATCC (Rockville, MD, USA). SH-SY5Y cells were cultured in DMEM-F12 medium from Lonza (VWR, Carnaxide, Portugal) with 20% fetal bovine serum (FBS) from Biowest (VWR, Carnaxide, Portugal) and 2 mM L-glutamine (Gibco); SK-N-BE(2) cells were cultured in RPMI 1640 medium from Biowest (VWR, Carnaxide, Portugal) with 10% FBS from Biowest (VWR, Carnaxide, Portugal); and HFF-1 cells were cultured in DMEM/F-12 from Lonza (VWR, Carnaxide, Portugal) supplemented with 10% FBS Biowest (VWR, Carnaxide, Portugal). All cells were maintained in a humidified incubator at 37°C with 5% CO₂ in the air.

The SK-N-BE(2) cells have a missense mutation at codon 135 (C135F), which converts cysteine to phenylalanine and gives rise to stable overexpressed p53 protein ¹²⁷.

Table 4. Human cell lines used in this work.

Cell line	Cell type	Growth properties	TP53 status
SH-SY5Y	Neuroblastoma	Mixed: adherent and suspension*	Wild-type ¹²⁷
SK-N-BE(2)	Neuroblastoma	Mixed: adherent and suspension*	Mutant (C135F) ¹²⁷
HFF-1	Foreskin fibroblasts	Adherent*	Wild-type ¹²⁸

*According to ATCC (American Type Culture Collection).

3.3. MTT assay

Cell viability as an indicator of the sensitivity of the cells to the compound LEM3 was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well microplates at a density of 7.5×10^3 (SH-SY5Y), 2.0×10^4 (SK-N-BE(2)) and 5×10^3 (HFF-1) cells/well in 75 μ L of culture medium in each well and allow the cells to adhere overnight. The next day, cells were treated with serial dilutions of the compound or only medium/medium with DMSO (controls) and the volume added was 75 μ L. Cells were then incubated at 37°C for 48h (T48 microplate) and a 96-well microplate seeded with cells containing only culture medium (T0 microplate), which serves as a control group, was also incubated. After incubation, cells were exposed to MTT solution (20 μ L per well; 5 mg MTT per mL of phosphate-buffered saline (PBS)), and incubated at 37°C for 2-4h, after this period, 100 μ L of solubilization solution was added per well with subsequent incubation overnight. In order to determine the half-maximal cell growth inhibitory concentration (GI_{50}) and 2-fold of the GI_{50} ($2GI_{50}$) values of compound LEM3, the absorbance of both microplates was measured by the microplate reader (Biotek Instruments Inc., Synergy MX, USA) at 590 nm. For the control group, the absorbance was set to 100% of the cell viability.

3.4. Cell cycle and apoptosis analysis

NB cells were seeded in 6-well plates at a density of 2.25×10^5 cells/well. After 24h incubation, cells were treated with DMSO only or with the GI_{50} or $2GI_{50}$ of LEM3 for 24 or 48h. For cell cycle analysis, the cells were first fixed with ice-cold 70% ethanol followed by the addition of 20 μ g/mL RNase A at 37°C. After 15 min at 37°C, 50 μ g/mL of propidium iodide (PI) from Molecular Probes (Alfagene, Carcavelos, Portugal) was further added and, after 30 minutes at room temperature or overnight at 4°C, flow cytometry analysis was performed. For apoptosis analysis, cells were suspended in binding buffer and incubated with 5 μ g/mL of Annexin V-FITC (Apoptosis Detection Kit I from BD Biosciences; Enzifarma, Porto, Portugal) and 5 μ g/mL of PI for 15 min at room temperature. Flow cytometry was used to analyse the cells. For the flow cytometry analysis, the AccuriTM C6 flow cytometer from BD Biosciences (Enzifarma, Porto, Portugal) and the CellQuest software from BD Biosciences (Enzifarma, Porto, Portugal) were used. FlowJo software (BD biosciences software) was used to identify and quantify the cell cycle phases, and also to quantify the analysis of annexin-positive cells.

3.5. Western blot analysis

To prepare the whole protein extracts from NB cells, the cells were lysed with RIPA buffer incorporating an EDTA-free protease inhibitor cocktail (Sigma-Aldrich, Sintra, Portugal). Protein extracts were quantified by the bicinchoninic acid (BCA) method (SigmaAldrich, Sintra, Portugal). After quantification, the proteins (30-40 µg) were loaded and separated based on their molecular weight (kDa) through a 10-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a Whatman nitrocellulose membrane from Protan (VWR, Carnaxide, Portugal) and finally, the membranes were blocked with 5% milk or 5% bovine serum albumin (BSA; for phosphorylated proteins) during 1h and probed with the respective antibody (antibodies are listed in Table 5). Briefly, the membrane was incubated with the primary antibody at 4°C overnight, then washed with Tris-buffered saline with Tween 20 (TBS-T) and incubated with a secondary antibody the next day for 2h at room temperature; GAPDH was used as a loading control. For the detection of the signal was used the ECL Amersham kit from GE Healthcare (VWR, Carnaxide, Portugal), and ChemiDoc™ XRS Imaging System from Bio-Rad Laboratories (Amadora, Portugal). Band intensities were quantified using the Image Lab software (Bio-Rad Laboratories) and the signal intensity corresponds to the respective loading and normalized to control (DMSO), set as 1. The Western blots presented are representative of at least two independent experiments.

Table 5. List of antibodies used in Western blot analysis.

Antigen	Type	Molecular weight of detected protein (kDa)	Final dilution	Supplier
Primary antibodies				
AIP4 (G-11)	Mouse monoclonal	106	1:100	Santa Cruz Biotechnology
ALK (F-12)	Mouse monoclonal	176	1:200	Santa Cruz Biotechnology
Bcl-2 (C-2)	Mouse monoclonal	26	1:100	Santa Cruz Biotechnology
GAPDH (6C5)	Mouse monoclonal	37	1:10 000	Santa Cruz Biotechnology

LIN-28 (6D1F9)	Mouse monoclonal	23	1:1000	Santa Cruz Biotechnology
MDM2 (SMP14)	Mouse monoclonal	90	1:200	Santa Cruz Biotechnology
N-MYC (B8.4.B)	Mouse monoclonal	67	1:1000	Santa Cruz Biotechnology
N-RAS (F155)	Mouse monoclonal	21	1:200	Santa Cruz Biotechnology
PARP-1 (F-2)	Mouse monoclonal	116 89 (cleaved product)	1:100	Santa Cruz Biotechnology
PUMA (G-3)	Mouse monoclonal	24	1:100	Santa Cruz Biotechnology
p21 (F-5)	Mouse monoclonal	21	1:100	Santa Cruz Biotechnology
p73 (E-4)	Mouse monoclonal	73	1:100	Santa Cruz Biotechnology
Survivin (EP2880Y)	Rabbit monoclonal	16	1:1000	Abcam
VEGF (C-1)	Mouse monoclonal	43	1:100	Santa Cruz Biotechnology
Secondary antibodies				
Anti-mouse HRP- conjugated	-	-	1:2500	Cell Signaling
Anti-rabbit HRP- conjugated	-	-	1:2500	Cell Signaling

3.6. miRNA assay

RNA analysis can provide crucial information regarding gene expression and this requires efficient extraction and purification to preserve RNA quality and integrity.

Table 6. List of miRNAs used in this work. Selected miRNAs are involved with p53 family proteins, namely TAp73, and/or biological processes in NB ^{98, 100, 113, 114, 129}. Expression levels were tested in the NB cell lines, SH-SY5Y and SK-N-BE(2).

miRNAs			
First panel			
miR-34a	miR-143	miR-200c	
Second panel			
Let-7i	miR-23a	miR-192	miR-200b

3.6.1. Treatment of the cells

SH-SY5Y and SK-N-BE(2) cells were seeded in 6-well plates at a density of $0,3 \times 10^6$ cells/well and allowed to adhere overnight, followed by treatment with DMSO only or with the GI_{50} or $2GI_{50}$ of LEM3 for 48h.

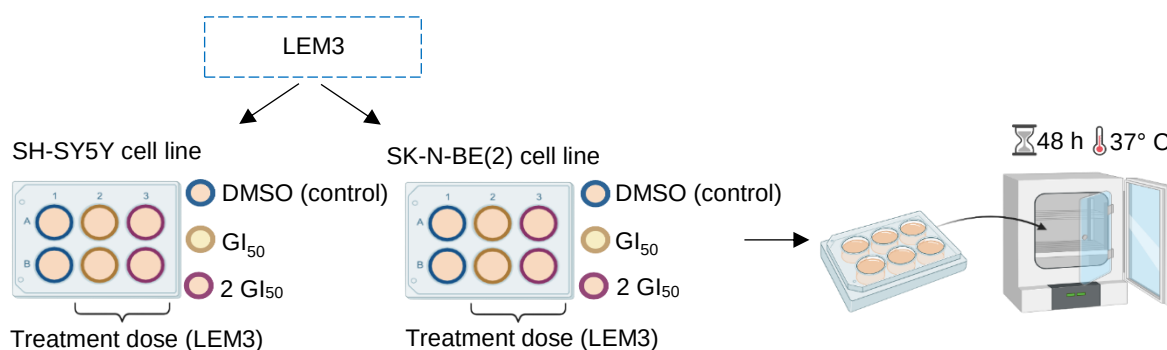


Figure 14. Schematic representation of treatment of the NB cells.

3.6.2. RNA extraction

RNA was isolated from NB cell lines after LEM3 treatment and in control group. The method for RNA extraction was based on cell lysis and denaturation, allowing the release of nucleic acids. Thus, total RNA from NB cells was extracted using 1 mL of TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA). Next, 0.2 mL of chloroform (Thermo Scientific) was added, mixed vigorously and centrifugation was performed at 12,000g for 15 minutes at 4°C. From the

result of the centrifugation, three phases are visible, an aqueous upper phase where RNA is present, an interphase consisting of DNA and finally, the organic phase consisting of proteins. After careful collection of RNA into a microcentrifuge tube, it was precipitated with 0.5 mL of isopropanol (Thermo Scientific), mixed vigorously and centrifuged at 12,000g for 10 minutes at 4°C. The RNA formed a pellet and the supernatant was removed and discarded. The precipitated RNA (pellet) was washed to remove impurities with 1 mL of 70% ethanol (Thermo Scientific)-free of RNases and centrifuged for 7,500g for 5 minutes at 4°C. Finally, the RNA pellet was resuspended in 20-50 μ L of diethylpyrocarbonate (DEPC)-Treated Water (Thermo Scientific).

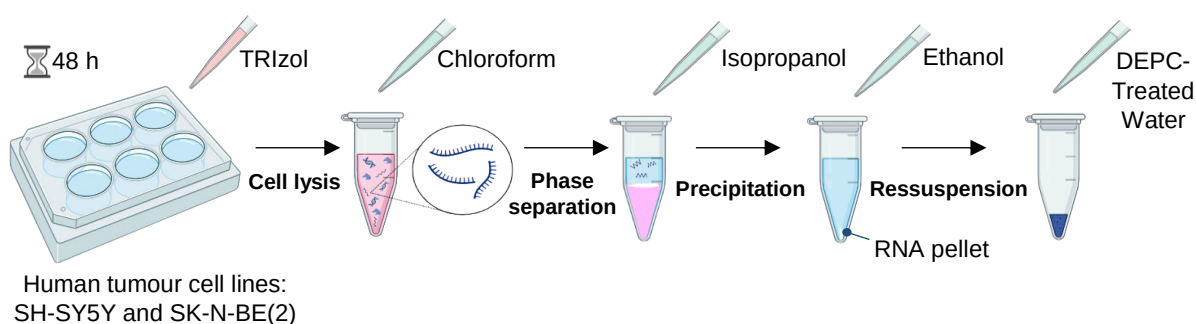


Figure 15. Schematic representation of the extraction of total RNA in NB cells.

3.6.3. Measuring RNA concentration and purity

RNA concentration and purity was measured using NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and NanoDrop software. Absorbance was measured at 260 nm to quantify RNA concentration. Ratio of 260/280 and 260/230 were determined to evaluate contamination by proteins or solvents. Stock RNA solution was diluted to 30 ng/ μ L.

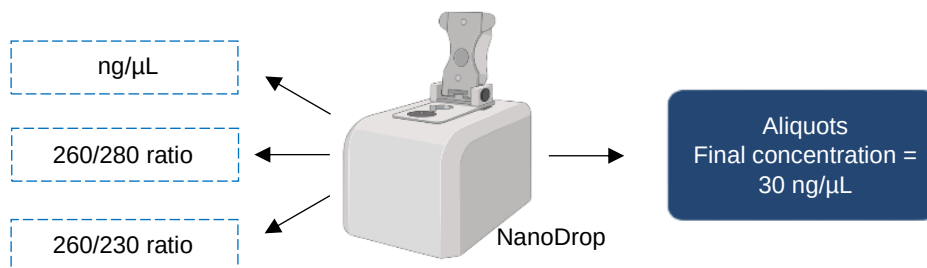


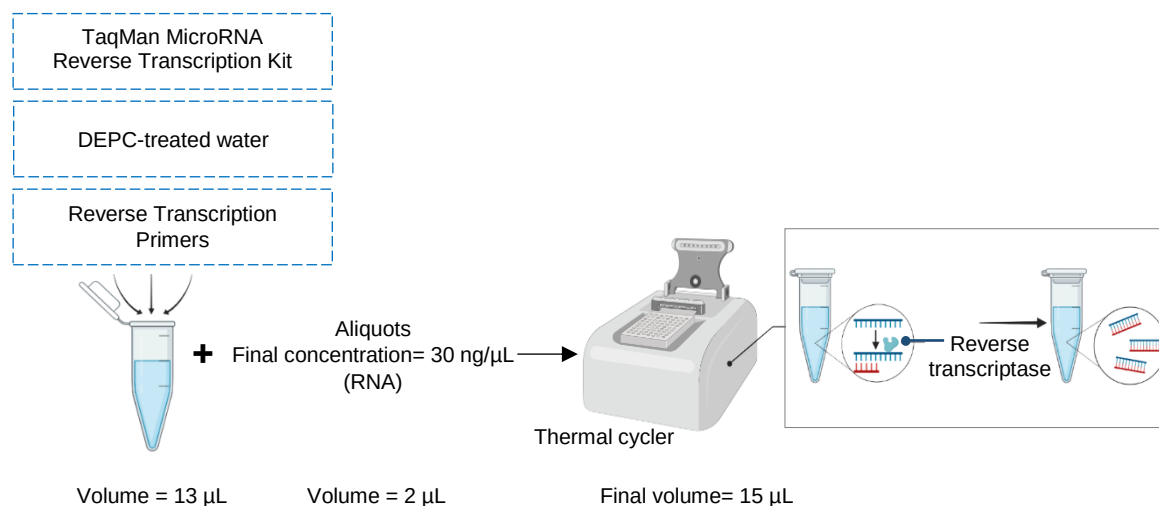
Figure 16. Schematic representation of RNA concentration and purity determination using NanoDrop.

3.6.4. Synthesis of cDNA and qPCR

For cDNA synthesis, a “RNA/primer” mix was prepared, including 60 ng RNA, stem-loop Reverse Transcription primers (primer let-7i, hsa-miR-23a, has-miR-34a, hsa-miR-143, hsa-miR-192, hsa-miR-200b, has-miR-200c and small nuclear RNA U6; Applied Biosystems) and TaqMan MicroRNA Reverse Transcription Kit, containing contains 10X RT buffer, dNTP mix w/dTTP, RNase inhibitor, and MultiScribe™ RT enzyme, (Applied Biosystems), according to the manufacture protocol. The reaction was synthesized in the thermocycler (T100™ Thermal Cycler, Bio-Rad).

To quantify miRNA expression, cDNA, a master mix (SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA)) and TaqMan miRNA assays (Applied Biosystems) for let-7i, hsa-miR-23a, has-miR-34a, hsa-miR-143, hsa-miR-192, hsa-miR-200b, has-miR-200c and small nuclear RNA U6 (Applied Biosystems), were mixed. The small RNA U6 was used as a reference gene. Water was used as a negative control. The experiment was performed in duplicates. The quantitative PCR (qPCR) reactions were performed in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Relative expression levels were calculated using the cycle of quantification (Cq) method, according to MIQE guidelines ¹³⁰.

(A)



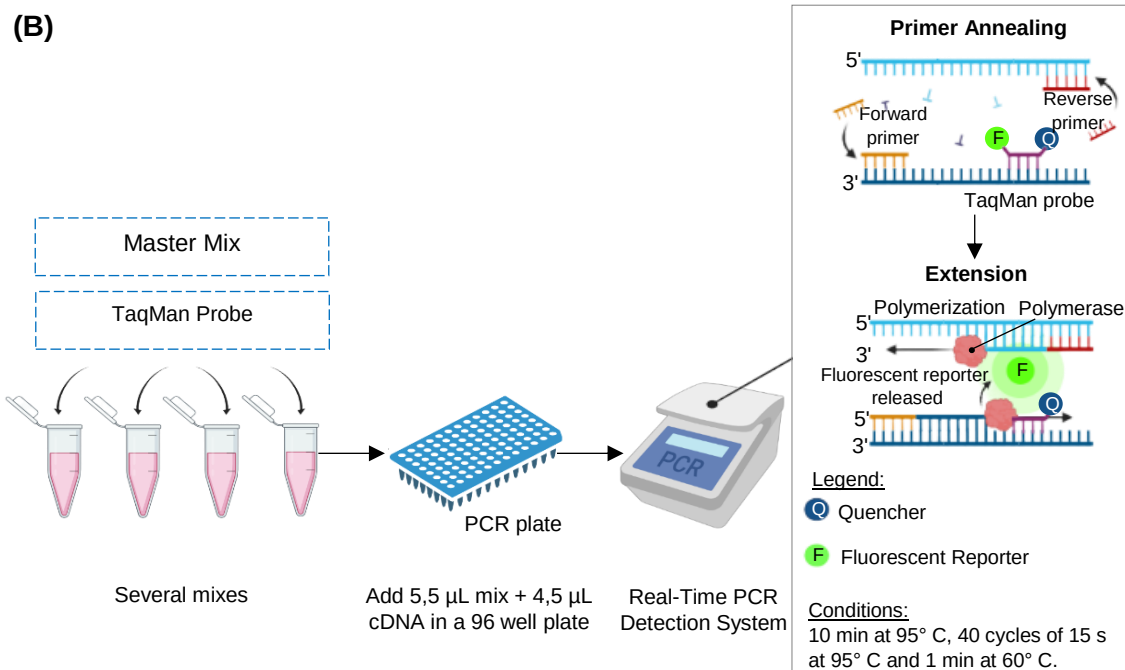


Figure 17. Schematic representation of (A) synthesis of cDNA and (B) qPCR using TaqMan miRNA assays.

3.7. Yeast phenotypic assay

Saccharomyces cerevisiae cells (strain CG379 *Mata*, *ade5*, *his7-2*, *leu2-112*, *trp1-289α*, *ura3-52*; Kil-O) from the Yeast Genetic Stock Center (University of California, USA), expressing human TAp73 alone and combined with human MDM2 or mutp53 (R273H) were obtained in previous studies^{119, 120}. To induce the expression of human proteins, yeast cultures (routinely grown in a selective medium) were diluted to 0.050 optical density at 600 nm (OD₆₀₀) in a selective galactose medium containing 2% (w/w) galactose (Sigma-Aldrich; Sintra, Portugal), 1% (w/w) raffinose (Acros Organics; JMGS, Odivelas, 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 mg/mL) except leucine and tryptophan. Yeast cells were incubated at 30 °C under continuous orbital shaking (170 rpm) with 0.1% DMSO or 5–20 µM of LEM3, for approximately 42h-48h. Yeast growth was analysed by counting the number of colony-forming units per mL (CFU/mL) after 2 days of incubation at 30°C on Sabouraud Dextrose Agar plates from Liofilchem (Frilabo, Porto, Portugal).

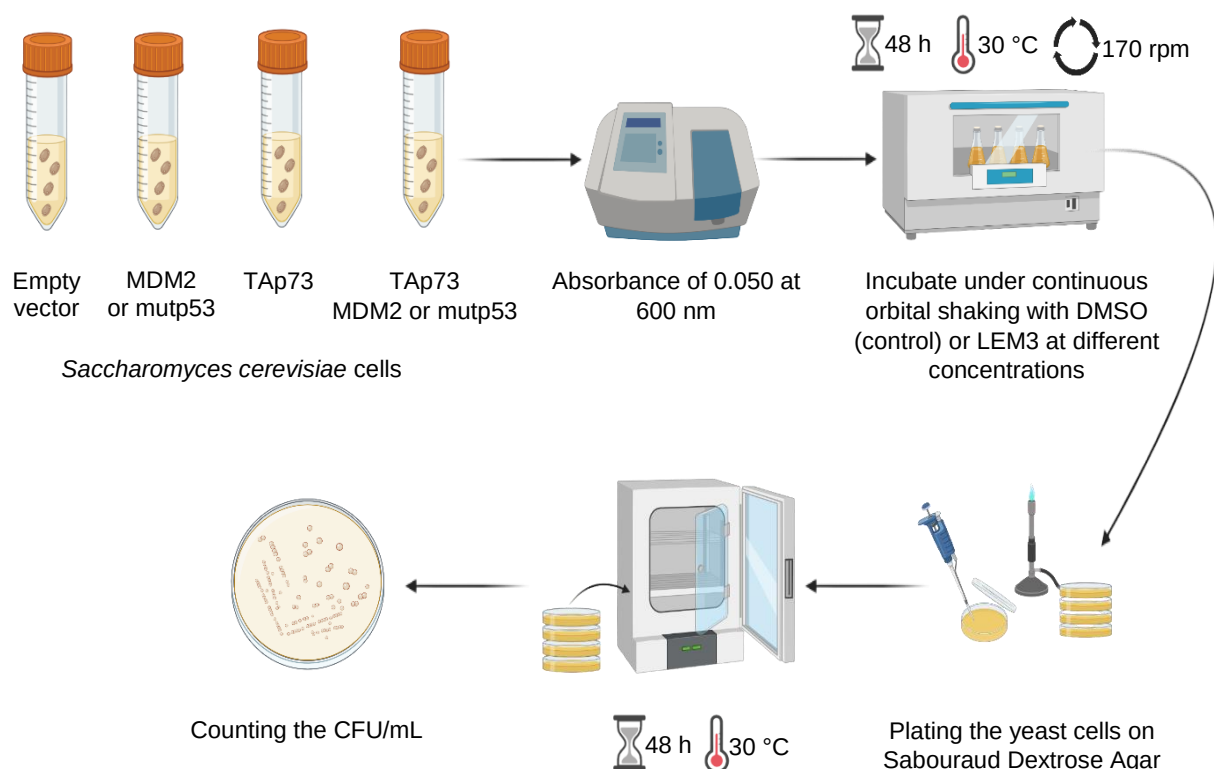


Figure 18. Schematic representation of yeast phenotypic assay.

3.8. Co-immunoprecipitation assay

The co-immunoprecipitation (co-IP) assay was performed to validate the molecular mechanism of action of LEM3 as an inhibitor of the TAp73 interaction with its negative regulators mutp53 and MDM2. Therefore, TAp73-mutp53 and TAp73-MDM2 interactions were studied in SK-N-BE(2) cells. For this assay, the Pierce Classic Magnetic IP/Co-IP Kit from Thermo Scientific (Dagma, Carcavelos, Portugal) was used. A total of 3.6×10^6 /flask SK-N-BE(2) cells were treated with 3GI_{50} of LEM3 or DMSO only for 16h. The cells were lysed in IP Lysis/Wash Buffer (Thermo Scientific) and protein concentration was determined with the Pierce™ BCA Protein Assay kit (Thermo Scientific).

After cell lysis and protein lysate separation, 500 µg of total protein was incubated with 10 µL of mouse monoclonal anti-p53 (DO-1) or mouse monoclonal anti-MDM2 (SMP14) or mouse immunoglobulin G (IgG, negative control) from Santa Cruz Biotechnology (Frilabo, Porto, Portugal), overnight at 4°C. The immunoprecipitation of the immunocomplexes was performed using Pierce™ Protein A/G magnetic beads (Thermo Scientific). After washing, the magnetic beads were resuspended in Lane Marker Sample Buffer (Thermo Scientific)

(containing 30 mM DTT) and boiled for 10 minutes at 96°C to elute bound proteins. The Western blot analysis was performed to detect TAp73, mutp53, MDM2 and GAPDH (was used as loading control) in the whole-cell lysate (input) and immunoprecipitated fractions (IP) as described above.

3.9. Statistical analysis

Data were statistically analysed using the GraphPad Prism software. Arithmetic means are given with a standard error of the mean (SEM). Gaussian distribution was tested by the Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Data was only considered to follow a normal distribution when passed both tests ($\alpha = 0.05$). For non-normal distribution data, or when $n < 5$, non-parametric tests were used to evaluate significant differences between samples, namely the Friedman test (> 2 groups) followed by uncorrected Dunn's multiple comparison test. When the data passed normality tests, the unpaired Student's *t*-test (between 2 groups) or the Two-way analysis of variance (ANOVA) (> 2 groups) were used. Appropriate statistical tests were applied to each dataset; $p < 0.05$ was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

4. RESULTS

In the present thesis, LEM3 was tested in human neuroblastoma cells in order to study its antitumour activity and validate its molecular mechanism of action as a TAp73-activating agent. Yeast cells were also used as an additional model system to further study the mode of action of the compound.

4.1. LEM3 has a TAp73-dependent growth inhibitory effect through enhancement of TAp73 transcriptional activity in neuroblastoma cells

The MTT assay was used to measure the cellular metabolic activity as an indicator of cell viability and proliferation. This colourimetric assay involves the conversion of yellow, water-soluble tetrazolium salt (MTT) into purple, water-insoluble formazan crystals by viable cells. Thus, the production of these crystals is directly proportional to the number of existing cells. The purple colour developed is a direct measure of the viability of the culture since its intensity translates into a higher number of metabolically active cells and, consequently, higher absorbency^{131, 132}.

The antiproliferative activity of LEM3 was evaluated by MTT assay, after 48h treatment in NB cell lines, SH-SY5Y and SK-N-BE(2). The selectivity of the compound for tumour cells was determined by comparing its anti-proliferative effect on these cells to that obtained in the normal human fibroblast cell line, HFF-1. Through the analysis of Figure 19, it can be verified that the GI₅₀ value of LEM3 was approximately ten times lower in SH-SY5Y cells (0.3 ± 0.003 μ M) and over five times lower in SK-N-BE(2) (0.5 ± 0.09 μ M), compared to HFF-1 cells (2.80 ± 0.14 μ M). The results also showed that LEM3 had potent antitumour activity in both NB cells, expressing wt (SH-SY5Y cells) or mutp53 (SK-N-BE(2) cells).

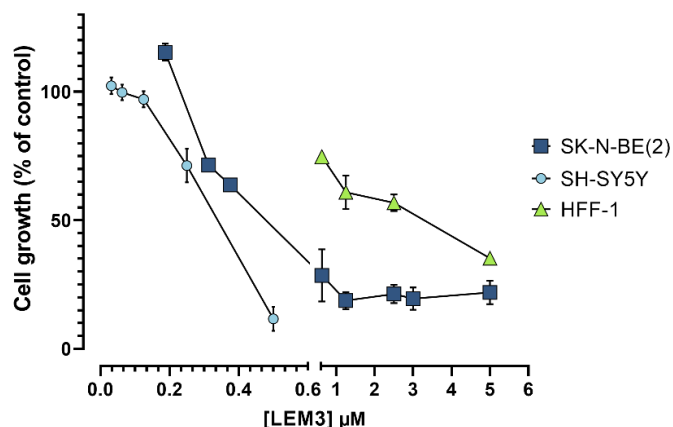


Figure 19. Dose-response curves for the growth inhibitory activity of LEM3 in SH-SY5Y, SK-N-BE(2) and HFF-1 cells. Results determined by MTT assay, after 48 h of treatment, with 0.03-5 μM LEM3. Data are mean $\pm$ SEM of four independent experiments.

With the confirmation of the LEM3 growth inhibitory effect on NB cells, we next evaluated whether this effect was associated with induction of apoptosis and/or cell cycle arrest.

In SH-SY5Y and SK-N-BE(2) cells, twice the GI_{50} ($2 \times \text{GI}_{50}$) concentration of LEM3 caused apoptosis, after 48h treatment (Figure 20A-B).

The effect of LEM3 on cell cycle arrest was also investigated in SH-SY5Y and SK-N-BE(2) cells (Figure 20C-D). After 24h of treatment, the 2GI_{50} LEM3 significantly induced cell cycle arrest in both cell lines. In SH-SY5Y cells, the growth inhibitory effect of 0.6 μM LEM3 was associated with G_0/G_1 phase cell cycle arrest (Figure 20C), while in SK-N-BE(2) cells, at 1 μM , LEM3 caused a G_2/M phase arrest (Figure 20D).

The results showed that LEM3 displayed a potent growth inhibitory effect, associated with cell cycle arrest and apoptosis, in SH-SY5Y and SK-N-BE(2) cells.

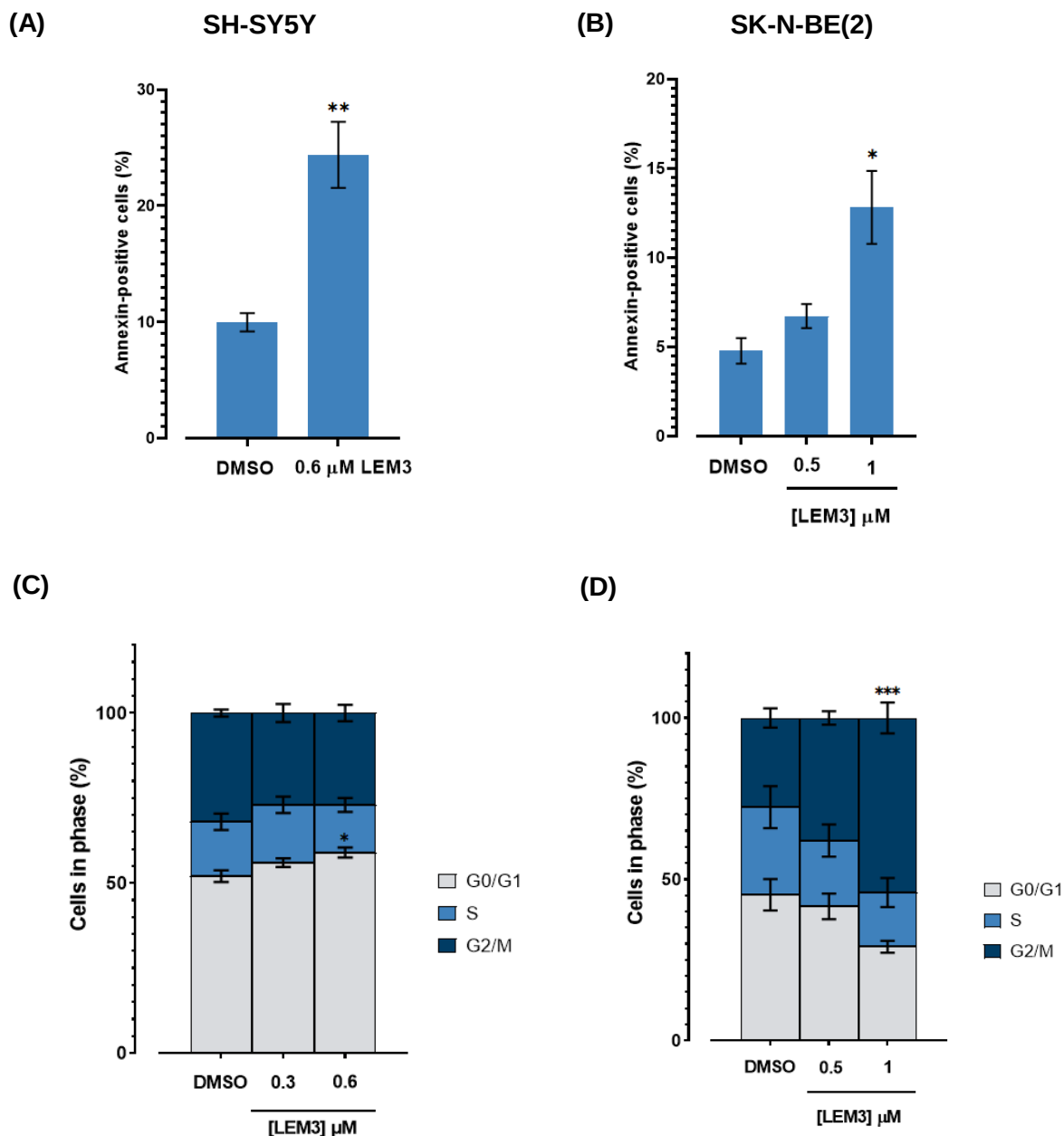


Figure 20. LEM3 induces apoptosis and cell cycle arrest in NB cells. (A) Effect with 0.6 μ M of LEM3 or DMSO on apoptosis induction in SH-SY5Y cells after 48h of treatment. (B) Effect with 0.5 and 1 μ M of LEM3 or DMSO on apoptosis induction in SK-N-BE(2) cells after 48h of treatment. Results are presented as percentage of annexin-positive cells. Data are mean $\pm$ SEM of at least two independent experiments and values significantly different from DMSO are indicated (* p < 0.05; ** p < 0.01). The unpaired student t-test was used. (C) Effect with 0.3 and 0.6 μ M of LEM3 or DMSO on cell cycle arrest in SH-SY5Y cells after 24h of treatment. (D) Effect with 0.5 and 1 μ M of LEM3 or DMSO on cell cycle arrest in SK-N-BE(2) cells after 24h of treatment. Results are presented as percentage of cells in each cell cycle phase. Data are mean $\pm$ SEM of at least two independent experiments and values significantly different from DMSO are indicated (* p < 0.05; *** p < 0.001). The two-way ANOVA analysis was used.

The antitumour activity of LEM3 was thereafter evaluated by Western blot analysis of the expression levels of distinct molecular targets related to the TAp73 pathway, in NB cells.

To assess whether LEM3-induced apoptosis was mediated by TAp73 activation, several transcriptional targets of TAp73 involved in apoptosis were assessed, including Bcl-2, PUMA and survivin. Of note, despite not being a TAp73 target, poly (ADP-ribose) polymerase-1 (PARP-1) is considered an important hallmark of apoptosis¹³³.

The results obtained showed that after 24h, the GI₅₀ and 2GI₅₀ concentrations of LEM3 increased the proteolytic cleavage of PARP-1, indicating stimulation of an apoptotic cell death, in SH-SY5Y and SK-N-BE(2) cells (Figure 21A-B).

Bcl-2 and survivin are anti-apoptotic proteins^{34, 37, 134}, and it was verified that LEM3, at 2xGI₅₀ concentration, decreased the expression levels of these proteins, in both cell lines (Figure 21A-B). PUMA is a pro-apoptotic protein⁶⁴, consistently, the results also revealed that 2xGI₅₀ concentration of LEM3 increased the expression levels of PUMA in both lines (Figure 21A-B).

Subsequently, the effect of LEM3 was investigated by analysis of the protein expression levels of a transcriptional target of TAp73 involved in cell cycle arrest, the p21³⁷. In SH-SY5Y cells, 0.6 µM LEM3 increased the protein levels of this cell cycle regulator p21, while in SK-N-BE(2) cells, there was also an increase of p21 levels for both concentrations tested (Figure 21C-D).

Moreover, it was observed that the protein levels of TAp73 increased for 0.6 µM LEM3 in SH-SY5Y cells. Consistently, in SK-N-BE(2) cells, LEM3 also increased the TAp73 levels in a dose-dependent manner (Figure 21C-D).

Altogether, the results indicated that, in SH-SY5Y and SK-N-BE(2) cells, LEM3 potentially enhanced TAp73 transcriptional activity by regulating the expression levels of TAp73 transcriptional targets, such as Bcl-2, survivin, PUMA and p21 (Figure 21). Furthermore, the increased expression levels of p21 corroborated a TAp73-dependent cell cycle arrest, and the modulation of the expression levels of Bcl-2, survivin and PUMA, a TAp73-dependent apoptosis.

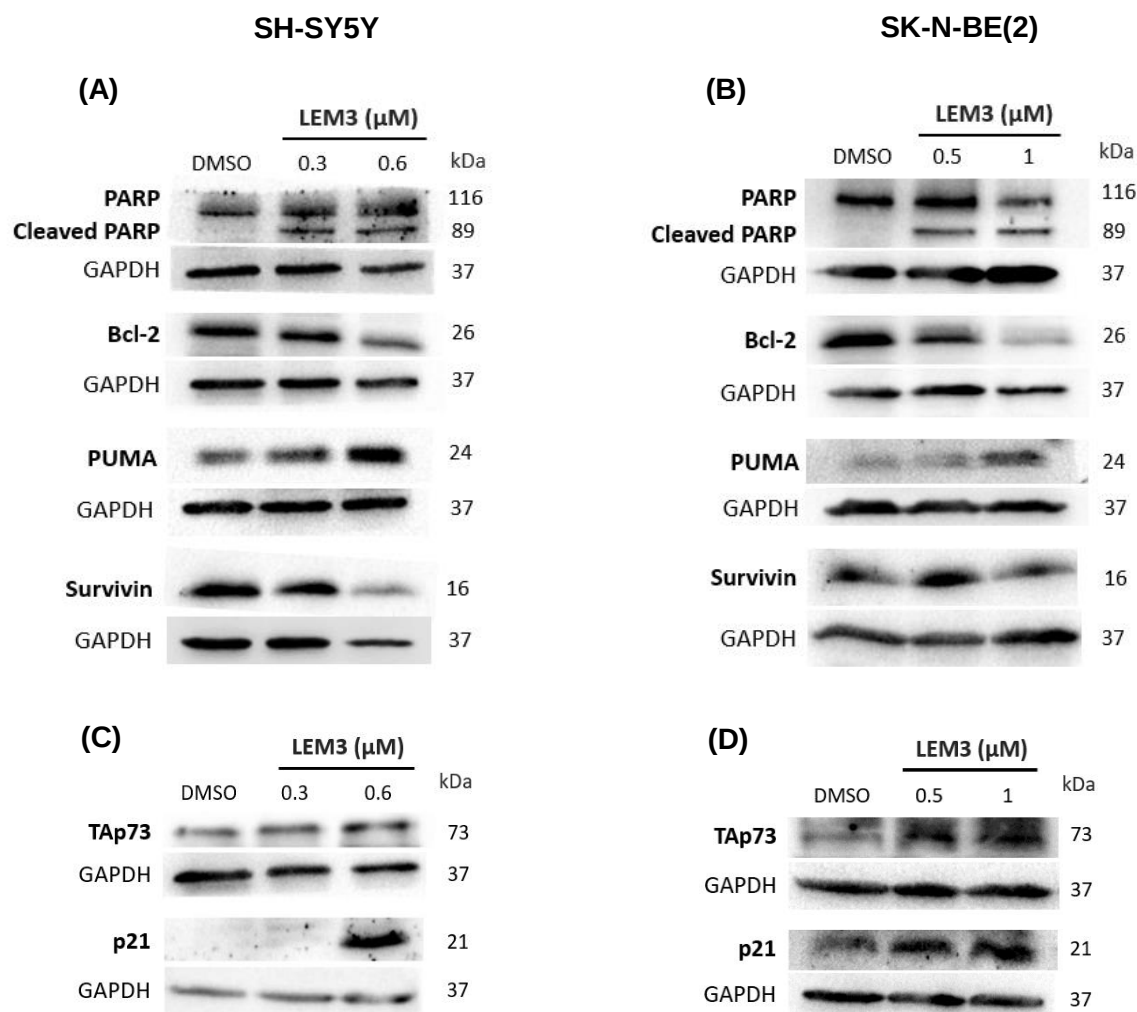


Figure 21. Effect of LEM3 on the protein expression levels of TAp73 and its target genes, and PARP cleavage, in NB cells. Western blot analysis of different apoptotic proteins was performed after 24h of treatment with 0.3 μ M (GI_{50}) and 0.6 μ M ($2GI_{50}$) of LEM3 or DMSO only in **(A)** SH-SY5Y and with 0.5 μ M (GI_{50}) and 1 μ M ($2GI_{50}$) of LEM3 or DMSO only in **(B)** SK-N-BE(2) cells. Western blot analysis of p21 and TAp73 expression levels was performed after 48 h of treatment with 0.3 μ M and 0.6 μ M of LEM3 or DMSO only in **(C)** SH-SY5Y and after 24h of treatment with 0.5 μ M and 1 μ M of LEM3 or DMSO only in **(D)** SK-N-BE(2) cells. Immunoblots are representative of at least two independent experiments. GAPDH was used as loading control.

Next, several markers of poor prognosis in NB were evaluated by Western blot after 48h treatment with LEM3, in SH-SY5Y and SK-N-BE(2) cells.

In particular, the protein levels of ALK (oncogene with roles in differentiation; ^{11, 135}), were downregulated in a dose-dependent manner in both cells (Figure 22A-B).

The results also showed a decrease in the protein expression levels of ITCH/AIP4 (a p73 inhibitor; ^{59, 60}), at 0.3 and 0.6 μ M LEM3 in SH-SY5Y cells. Whereas, in SK-N-BE(2) cells, there was a significant decrease of ITCH/AIP4 at 1 μ M LEM3 (Figure 22A-B).

In SH-SY5Y cells, it was observed that 0.3 and 0.6 μ M LEM3 increased the protein levels of MDM2 (a negative regulator of p53 family proteins; ⁴⁸) (Figure 22A).

As mentioned above, the upregulation of *MYCN* expression is an important biomarker in the progression of high-risk NB ¹¹. In SK-N-BE(2) cells the protein levels of N-MYC were downregulated, in a dose-dependent manner, by LEM3 (Figure 22B). In these cells, 1 μ M, LEM3 also downregulated the protein levels of VEGF (involved in angiogenesis and associated with advanced-stage NB tumours; ¹³) (Figure 22B).

Another poor prognostic marker is LIN28B, which is strongly involved in the development of high-risk NB ¹¹. Interestingly, LEM3 also decreased the protein expression levels of LIN28B, at 0.6 μ M, in SH-SY5Y cells (Figure 22A), and at 0.5 and 1 μ M, in SK-N-BE(2) cells.

Furthermore, in SH-SY5Y cells, this compound caused a dose-dependent decrease in N-RAS protein levels, which is an EMT marker associated with aggressive NB; ^{11, 136} (Figure 22A). In SK-N-BE(2) cells, there was a visible decrease in N-RAS levels at 1 μ M LEM3 (Figure 22B).

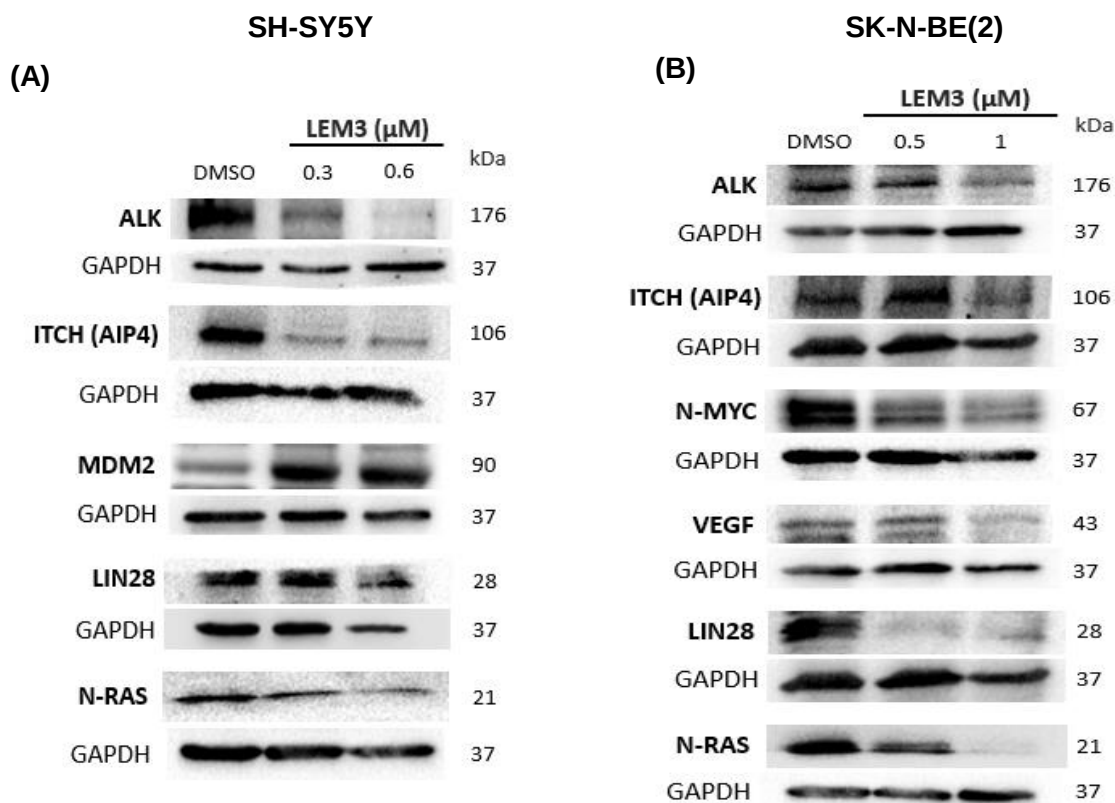


Figure 22. Effect of LEM3 on the protein expression levels of poor prognostic markers of NB. Western blot analysis was performed after 48 h treatment with 0.3 μ M (GI_{50}) and 0.6 μ M ($2GI_{50}$) of LEM3 or DMSO only in **(A)** SH-SY5Y and with 0.5 μ M (GI_{50}) and 1 μ M ($2GI_{50}$) of LEM3 or DMSO only in **(B)** SK-N-BE(2) cells. Immunoblots are representative of at least two independent experiments. GAPDH was used as loading control.

4.2. LEM3 potentially inhibits the TAp73-MDM2 interaction in yeast and human tumour cells

As mentioned, MDM2 is known to inhibit the transcriptional activity of TAp73 and is often overexpressed in NB ^{48, 62}. By binding to TAp73, MDM2 inhibits its activity ⁴⁸, thus, a potential disruption of this protein-protein interaction by LEM3 may explain the activation of TAp73 activity.

To understand whether LEM3 could also inhibit the TAp73-MDM2 interaction, as observed with LEM2 ¹¹⁹, we used a yeast phenotypic assay previously developed by our group ¹¹⁹. This assay used yeast cells transformed with the empty vector (used as a control yeast), yeast cells expressing only MDM2 or TAp73, and yeast cells co-expressing TAp73 and MDM2. In this assay, the growth of treated co-transformed yeasts is compared to that of co-transformed yeasts treated with solvent (DMSO) and yeasts only expressing TAp73/MDM2. Since TAp73 induces a growth arrest proportional to its activity in co-transformed yeasts, which is abolished by MDM2, an inhibitor of the TAp73-MDM2 interaction will reestablish the TAp73-induced yeast growth inhibition ¹¹⁹.

The results revealed no significant differences between the growth of yeast cells transformed with the empty vector and expressing MDM2/TAp73 alone treated with LEM3 and DMSO. However, 20 μ M LEM3 decreased the growth of yeast cells co-expressing TAp73 and MDM2, which suggested that LEM3 potentially inhibited the TAp73 interaction with MDM2 (Figure 23).

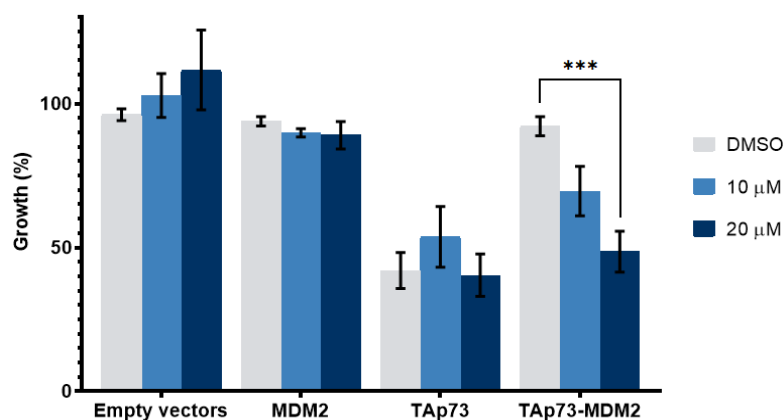


Figure 23. LEM3 inhibits the TAp73-MDM2 interaction in yeast cells co-expressing TAp73 and MDM2. Effect of LEM3 on the growth of control yeast (empty vectors), yeast cells expressing MDM2 or TAp73 alone, and yeast cells co-expressing TAp73 and MDM2, was evaluated using the yeast phenotypic assay, after 48 h treatment with 10 and 20 μ M LEM3 or DMSO only. Results were plotted setting the growth of untreated control yeast as 100%. Data are mean $\pm$ SEM of at least three independent experiments; values significantly different from DMSO: *** p < 0.001 (two-way ANOVA with Dunnett's multiple comparison test).

To validate this potential disruption of the TAp73-MDM2 interaction by LEM3, observed in yeast cells, a co-IP assay was performed in SK-N-BE(2) cells (Figure 24A). As expected, in these cells, LEM3 inhibited the interaction between TAp73 and MDM2. In fact, at 1.5 μ M there was a decrease in the amount of TAp73 co-immunoprecipitated with MDM2, indicating the disruption of the TAp73-MDM2 interaction by the compound (Figure 24A-B).

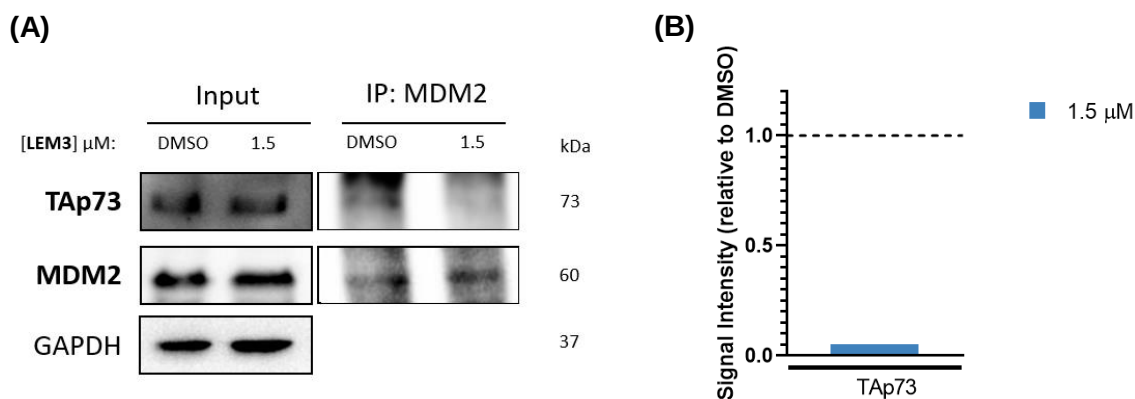


Figure 24. LEM3 inhibits the TAp73-MDM2 interaction in SK-N-BE(2) cells. **(A)** Co-IP was performed in SK-N-BE(2) cells treated with 1.5 μ M ($3GI_{50}$) of LEM3 or DMSO only for 16 h. SK-N-BE(2) cells lysates were immunoprecipitated (IP) with anti-MDM2 (IP:MDM2) antibody, followed by immunoblotting with anti-TAp73 and anti-MDM2 antibodies; whole cell lysate (input). GAPDH was used as loading control. Immunoblots are representative of one independent experiment (ongoing work). **(B)** In the graph, quantification of protein expression levels relative to DMSO is shown (set as 1); data are mean $\pm$ SEM, n=1 (ongoing work).

Altogether, these data corroborated a potential interference of LEM3 with the TAp73-MDM2 interaction.

4.3. LEM3 potentially inhibits the TAp73-mutp53 interaction in yeast and human tumour cells

Since mutp53 can also interact with TAp73, blocking the transcriptional activity of TAp73⁵⁰, and being its appearance in tumours highly associated with relapsed cases of NB, it became relevant to evaluate the effect of LEM3 on the mutp53-TAp73 interaction. For that, we started by studying the interference of LEM3 on the TAp73-mutp53 interaction using a yeast phenotypic assay followed by validation of the results by co-IP in a NB cell line.

Similarly to that described above, in the yeast phenotypic assay, it was evaluated the effect of LEM3 on the growth of control yeast (transformed with the empty vectors), yeast cells expressing only mutp53-R273H (a highly prevalent mutp53 form associated with gain of function activity through interaction with TAp73 in human tumour cells⁷¹) or TAp73, and yeast cells co-expressing TAp73 and mutp53-R273H. The results showed that the compound had no effect on the growth of control yeast and yeast cells expressing mutp53 or TAp73 alone. In the case of yeast co-expressing TAp73 and mutp53, 5 and 10 μ M LEM3 inhibited the growth of yeast cells, suggesting a restoration of the TAp73 transcriptional activity due to a disruption of its interaction with mutp53 (Figure 25).

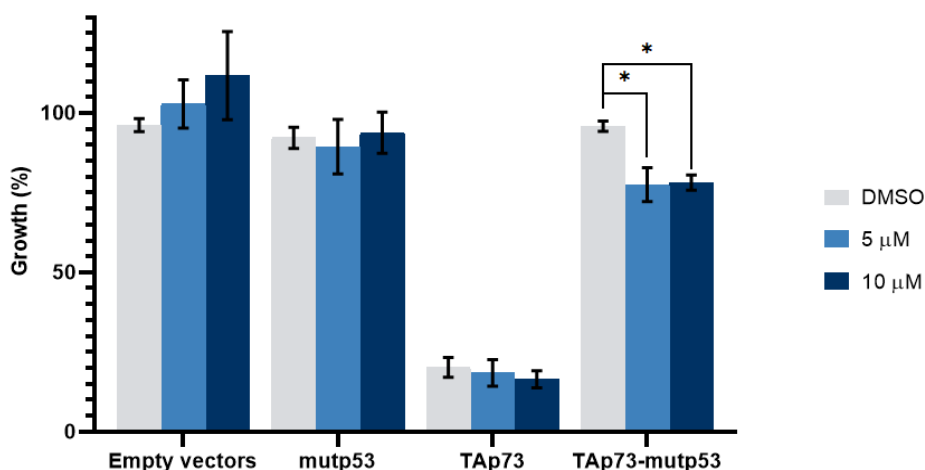


Figure 25. LEM3 inhibits the TAp73-mutp53 interaction in yeast cells co-expressing TAp73 and mutp53. Effect of LEM3 on the growth of control yeast (empty vectors), yeast cells expressing mutp53-R273H or TAp73, and yeast cells co-expressing TAp73 and mutp53-R273H, was evaluated by yeast phenotypic assay, after 48 h treatment with 5 and 10 μ M LEM3 or DMSO only. Results were plotted setting the growth of untreated control yeast as 100%. Data are mean $\pm$ SEM at least three independent experiments; values significantly different from DMSO: * $p < 0.05$ (two-way ANOVA with Dunnett's multiple comparison test).

The co-IP assay was performed to confirm that LEM3 interferes with the interaction of TAp73 with mutp53, in the mutp53-expressing human NB cell line, SK-N-BE(2) (Figure 26). The results confirmed that 1.5 μ M LEM3 inhibited the interaction between TAp73 and mutp53, since it visibly decreased the amount of TAp73 co-immunoprecipitated with mutp53 (Figure 26A-B).

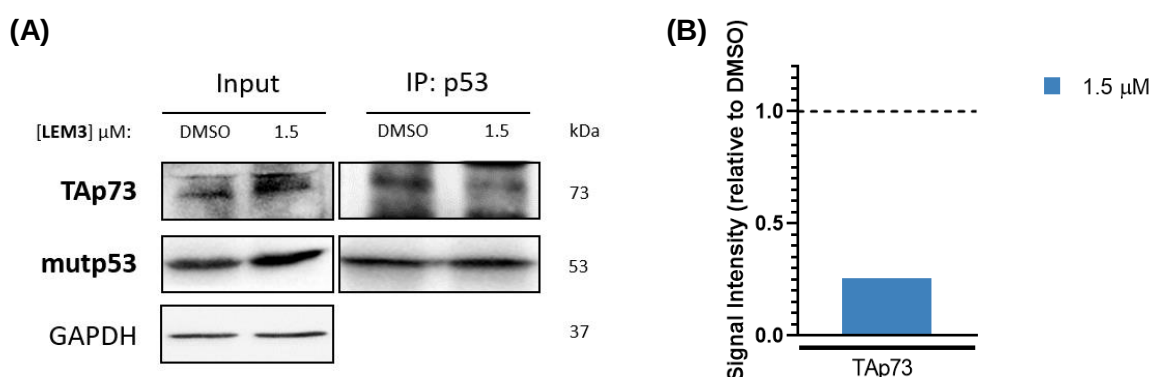


Figure 26. LEM3 inhibits the TAp73-mutp53 interaction in SK-N-BE(2) cells. (A) Co-IP was performed in SK-N-BE(2) cells treated with 1.5 μ M (3GI₅₀) of LEM3 or DMSO only for 16h. SK-N-BE(2) cells lysates were immunoprecipitated (IP) with anti-p53 (IP:p53) antibody, followed by immunoblotting with anti-TAp73 and anti-p53 antibodies; whole cell lysate (input). GAPDH was used as loading control. Immunoblots are representative of one independent experiment (ongoing work). **(B)** In the graph, quantification of protein expression levels relative to DMSO is shown (set as 1); data are mean $\pm$ SEM, n=1 (ongoing work).

These data corroborated a potential interference of LEM3 with the TAp73-mutp53 interaction.

Collectively, the results indicated that LEM3 is a potential dual inhibitor of the TAp73 interaction with MDM2 and mutp53.

4.4. Impact of LEM3 on selected miRNAs involved in TAp73 pathway and biological processes in neuroblastoma

We next study whether LEM3 changes the expression levels of relevant miRNAs involved in the p73 pathway or biological processes in NB, namely the expression of miR-34a, miR-143, mir-200c, let-7i, mir-23a, miR-192 and miR-200b.

Regarding the selected miRNAs, results show that miR-34a, miR-143, mir-200c, let-7i, mir-23a and miR-192 are expressed with Cq values between 22.86 and 34.52 in SH-SY5Y cells (Figure 27A-B). However, miR-200b is undetected or expressed at very low levels, with the Cq values higher than 35 cycles and thus not considered.

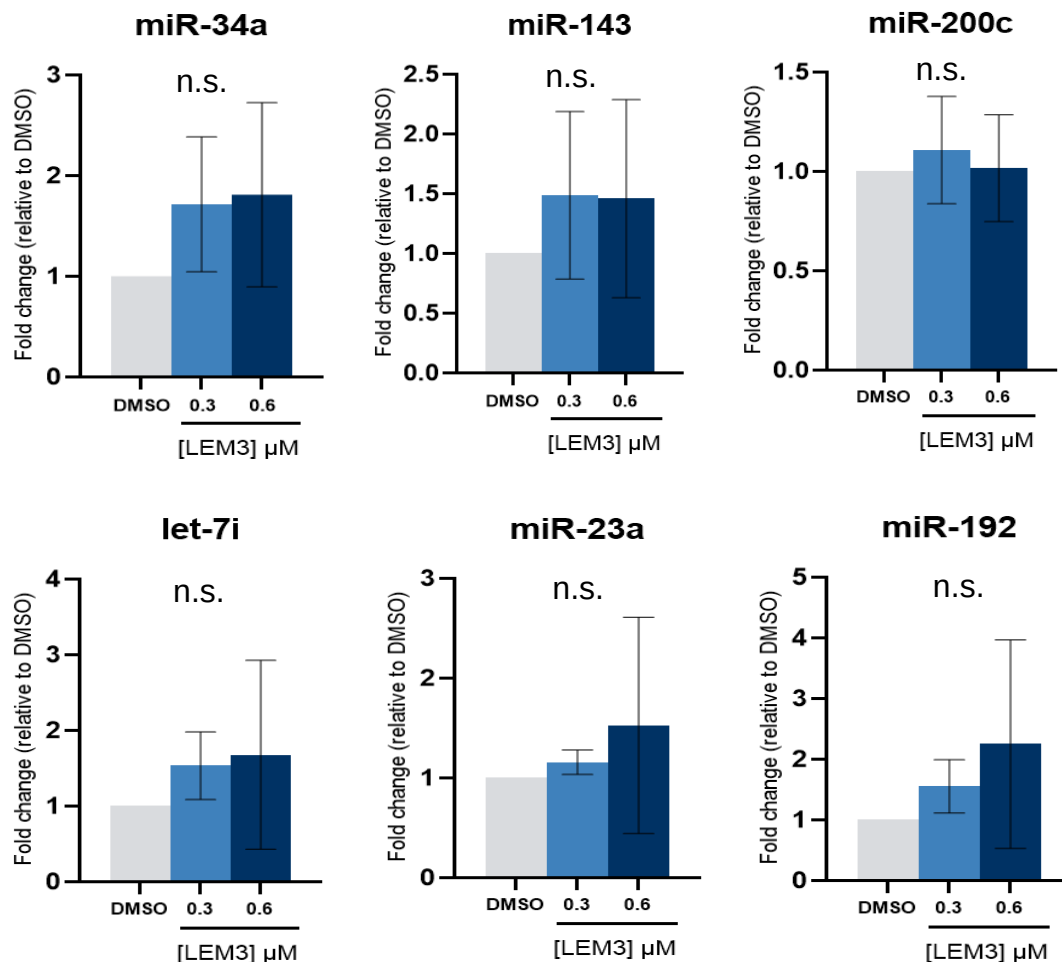


Figure 27. Interference of LEM3 in miRNAs expression levels, in SH-SY5Y cells. Expression levels of miR-34a, miR-143 and miR-200c (n=6) and let-7i, miR-23a and miR-192 (n=3) in SH-SY5Y cells after 48 h of treatment with 0.3 μM (GI_{50}) and 0.6 μM ($2GI_{50}$) of LEM3 or DMSO only. Fold change is relative to DMSO. Small nuclear RNA U6 was used as a reference gene. Values are representative of 3 to 6 independent experiments (mean $\pm$ SEM; Friedman test, followed by uncorrected Dunn's multiple comparison test; n.s.: non-significant).

Regarding SK-N-BE(2) cells, miR-143, mir-200c, let-7i, mir-23a and miR-192 are expressed with Cq values between 23.21 and 32.95 (Figure 28), while miR-34a and miR-200b are undetected or expressed at very low levels, with the Cq values higher than 35 cycles.

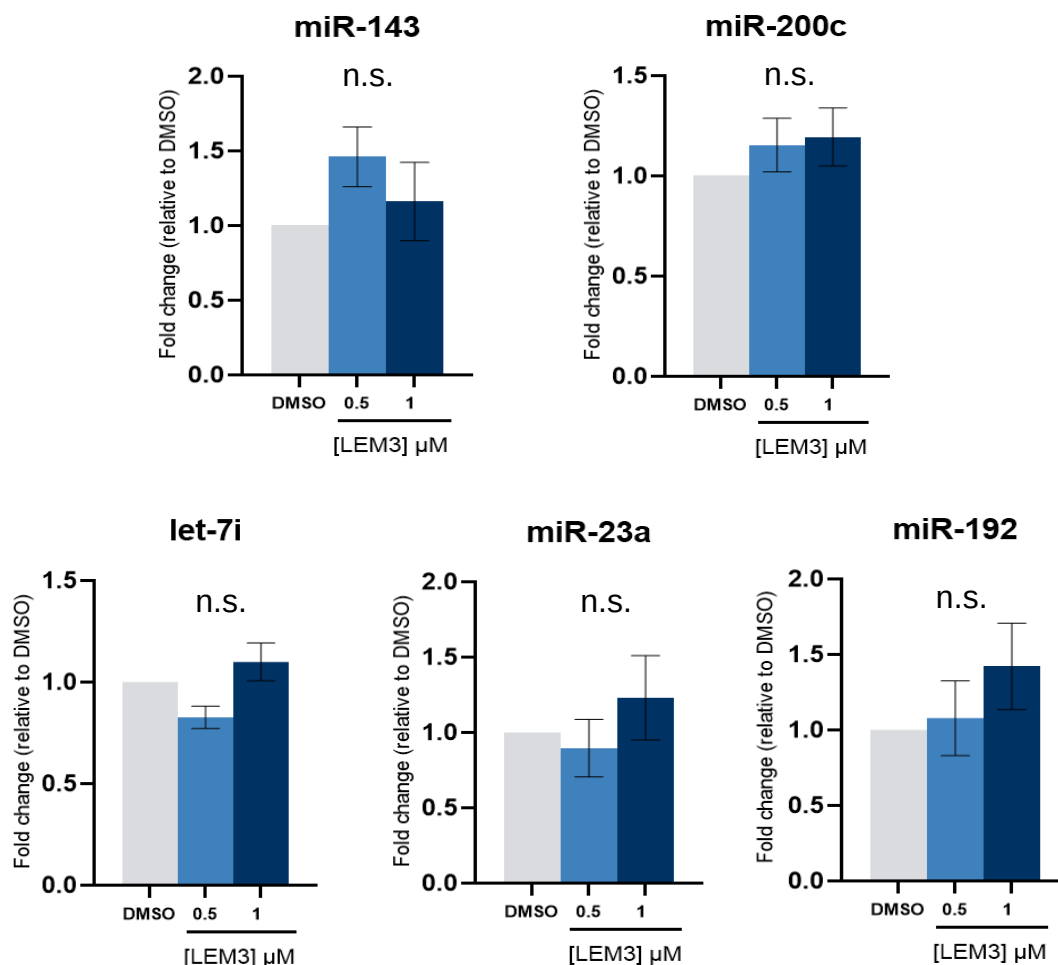


Figure 28. Interference of LEM3 in miRNAs expression levels, in SK-N-BE(2) cells. Expression levels of miR-143 and miR-200c (n=6) and let-7i, miR-23a and miR-192 (n=3) in SK-N-BE(2) cells after 48 h of treatment with 0.5 μM (GI₅₀) and 1 μM (2GI₅₀) of LEM3 or DMSO only. Fold change is relative to DMSO. Small nuclear RNA U6 was used as a reference gene. Values are representative of 3 to 6 independent experiments (mean±SEM; Friedman test, followed by uncorrected Dunn's multiple comparison test; n.s.: non-significant).

Although the majority of the selected miRNAs were expressed in both SH-SY5Y and SK-N-BE(2) cells, no significant changes in their expression levels were observed following LEM3 treatment, compared with control. In summary, LEM3 had no effect on these TAp73-related miRNAs.

5. DISCUSSION

Even with the currently available therapeutic options based on risk stratification, including surgery, chemotherapy, radiotherapy, autologous stem cell transplantation and immunotherapy, NB cells often develop resistance mechanisms which can lead to high-risk patients with low survival rate (with a 5-year survival rate of less than 50%) and relapses ^{12, 14}. Several new therapies have appeared depending on the therapeutic target under study (usually proteins), but despite the emergence of numerous compounds, there are still many underlying limitations. Hence, efficient therapeutic alternatives against NB are urgently required ^{10, 12, 14}.

The deregulation of the p53 family pathway plays a critical role in the tumourigenesis of NB ¹³⁷. Particularly, a major focus has been placed on the p73 pathway for the development of new anticancer drugs ¹³⁷. In fact, TAp73 has a major role in the formation, development and differentiation of NB ^{33, 35}. In addition to its similar tumour suppressive activity to p53, TAp73 downregulates *MYCN*, one of the most important genetic signatures of poor prognosis in NB ^{34, 40, 44}. However, TAp73 is commonly inactivated in NB by interaction with MDM2 (overexpressed in NB and strongly related to poor prognosis) ^{48, 62} and mutp53 (associated with relapse and acquisition of therapeutic resistance in NB) ^{50, 76}.

Our group identified the compound LEM2 with potent antitumour activity in NB cells, either as a single agent or in combination with current chemotherapy ¹¹⁹. LEM2 was considered an activator of TAp73 through inhibition of its interaction with the negative regulators, MDM2 and mutp53 ¹¹⁹. However, the low aqueous solubility of LEM2 has limited *in vivo* antitumour studies. Based on this, a small library of LEM2-derivatives was synthesised and the dibrominated xanthone LEM3 was selected, due to its improved solubility. With this work, we report the antitumour activity and potential molecular mechanism of action of LEM3 in NB cells.

The antiproliferative activity of LEM3 increased in wtp53- (SH-SY5Y) expressing tumour cell line, when compared to the carbaldehydic xanthone LEM2 ¹¹⁹. LEM3 also showed selectivity to NB cells, with an inhibitory activity ten- (in case of SH-SY5Y cells) and five- (in case of SK-N-BE(2) cells) fold lower in the normal human fibroblast cell line, HFF-1.

By analysis of cell cycle progression and Annexin-V staining, it was observed that the growth inhibitory effect of LEM3 was associated with induction of a G₀/G₁ phase cell cycle arrest in SH-SY5Y cells and G₂/M phase arrest in SK-N-BE(2) cells, as well as apoptosis in both cell lines. These results are supported by an increase in the protein levels of TAp73 and respective target genes, including of p21 cell cycle regulator. In fact, in NB patients, decreased p21 expression levels appear to be associated with poor outcomes ¹³⁸. Other TAp73 targets

involved in apoptosis corresponded to the pro-apoptotic protein PUMA and the anti-apoptotic proteins Bcl-2 and survivin. Regarding PUMA, which is a member of the BCL-2 family, it is known that its activity can be inhibited by binding to Bcl-2^{37, 64}. Bcl-2 is highly expressed in NB and its expression is correlated with poor prognosis, such as *MYCN*-amplification^{139, 140}. Consistently the expression of survivin is also associated with high-risk tumours¹⁴¹. Therefore, Bcl-2 and survivin are useful prognostic markers in NB. Additionally, the induction of apoptosis was reinforced by increased PARP-1 cleavage. This nuclear enzyme is involved in DNA damage repair pathways and cleavage of this protein is an indication of apoptosis¹³³.

Several other markers with key roles in tumour progression and poor prognosis in NB, such as ALK, ITCH, N-MYC, VEGF, LIN28B and N-RAS, were found downregulated by LEM3 in NB cells. In particular, mutations in the *ALK* gene are present in most patients with familial NB^{11, 12}, and SH-SY5Y cells have the ALK(F1174L) mutation, which is known to cause acquired resistance to the crizotinib (*ALK* inhibitor)¹⁴². Hence, LEM3 decreased the protein expression levels of ALK in both NB cell lines tested, but the compound has potential use for the treatment of patients with this type of *ALK* mutation. ITCH is also found overexpressed in NB, being a p73 inhibitor; when ITCH is inhibited, induction of apoptosis occurs, as well as suppression of tumour cell proliferation¹⁴³. Additionally, the amplification of the *MYCN* oncogene represents one of the most relevant genetic alterations in NB¹². Several studies in NB tumours revealed that when this marker is overexpressed, TAp73 is downregulated, and consistently, TAp73 overexpression downregulates *MYCN*^{40, 44, 45}. Regarding VEGF, it is an important regulator of tumour angiogenesis and increased expression of this marker indicates poor prognosis in NB^{13, 27}. In addition, VEGF overexpression seems to be associated with *MYCN* amplification in NB¹⁴⁴. LIN28B is another crucial marker frequently overexpressed in high-risk NB and an inhibitor of the let-7 miRNA¹³. The inhibition of let-7 increases the expression of the N-MYC protein¹³. On the other hand, the N-RAS inhibition is pointed out as another important therapeutic target, as GOF mutations in this oncogene are related to unfavourable outcomes¹¹. Altogether, these data not only establish a correlation between the stimulation of TAp73 protein levels and inhibition of poor prognosis markers in NB but also reinforce the great antitumor potential of this compound, namely highlighting its antiangiogenic activity and enhancer of differentiation.

LEM3 further increased the protein levels of another poor prognostic marker in high-risk NB patients, MDM2⁴⁸. This marker is overexpressed in NB and has affinity to TAp73, suppressing its transcriptional activity⁴⁸. Although there is an increase of this marker in LEM3-

treated NB cells, this is consistent with an increase in TAp73 levels and subsequent transcriptional activity, since MDM2 is a TAp73 target.

To further elucidate the molecular mechanism of TAp73 activation by LEM3, we tested the ability of this compound to also disrupt the TAp73-MDM2 and TAp73-mutp53 interactions as observed with LEM2. For that, a yeast phenotypic assay was used to study the effect of LEM3 on TAp73-MDM2 interaction. The results indicated that LEM3 was able to release TAp73 from the MDM2-inhibitory effect, re-establishing the TAp73-induced yeast growth arrest. In order to confirm the results obtained in yeast cells, the inhibition of TAp73-MDM2 interaction by LEM3 was checked in SK-N-BE(2) NB cells by a co-IP assay. In accordance with the yeast assay, in these cancer cells, the compound led to a decrease in the amount of TAp73 co-immunoprecipitated with MDM2, supporting an inhibition of the TAp73-MDM2 interaction by LEM3. Likewise, we used the yeast assay to confirm the inhibitory effect of LEM3 on the TAp73-mutp53, a result also validated in SK-N-BE(2) NB cells by co-IP assay. Altogether, the results obtained in this work indicated that LEM3 restores the TAp73 transcriptional activity by disrupting its interaction with the negative regulators MDM2 and mutp53.

As TAp73, the tumour suppressor miR-34a is also implicated in neuronal differentiation¹⁰⁸, as well as in apoptotic and anti-metastatic behaviour by repressing several oncogenes¹¹³. miR-34a is targeted by TAp73¹⁰⁸. This miRNA plays an important role in NB, inducing cell cycle arrest and apoptosis, and reducing angiogenesis. Examples of targets of miR-34a are Bcl-2 and MYCN^{108, 109}. TAp73 drives the expression of miR-34a, as such it regulates cellular functions, like neuronal differentiation, at least in part, through miR-34a¹⁰⁸. Our results indicated that miR-34a was expressed in the SH-SY5Y cells, but at very low levels or not expressed in SK-N-BE(2) cells. These results are in line with the study conducted by Li and Chen¹⁴⁵, in which the authors reported the low expression levels of miR-34a in paediatric NB tissues, and its overexpression in SH-SY5Y cells could inhibit cell proliferation, migration, and invasion¹⁴⁵. The residual expression levels of miR-34a in SK-N-BE(2) cells observed in this work are also corroborated by a study of Welch *et al.*¹⁴⁶, in which the authors demonstrated an expression level of this miRNA 50-fold lower compared to another NB cell line, KELLY.

A study by Ying *et al.* (2020) demonstrated, for the first time, that miR-143 functions as a tumour suppressor in NB, being significantly downregulated in NB cells compared to normal human dorsal ganglia¹⁴⁷. TAp73 regulates the processing of miRNA-143¹⁰⁰. In our results, miR-143 was expressed in SH-SY5Y and SK-N-BE(2) cells, which was corroborated by a previous work¹⁴⁷. Although an increase in miR-143 expression levels was observed in LEM3-

treated conditions compared to vehicle, in SK-N-BE(2) cells, but the high deviation values could not allow us for more confident conclusions. This deviation was even more pronounced in SH-SY5Y cells. More experiments should therefore be performed to clarify these results.

The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) is also a common target of the p53 family proteins, and all members of the miR-200 family are important mediators of tumour suppression^{100, 113}. The miR-200 family is implicated in the regulation of various cancer-related processes, such as proliferation, apoptosis, EMT, migration and invasion¹⁴⁸. The miR-200b and miR-200c (selected for this work) had never been tested in SK-N-BE(2) cells, whereas a study revealed that these miRNAs were expressed in SH-SY5Y cells¹⁴⁹. The results obtained in this work revealed that while miR-200c is expressed in SK-N-BE(2) cells, miR-200b was not detected in these cells.

The miR-192 is a prognostic marker of relapse in NB⁹⁹ and is a TAp73 target¹⁰⁰. This miRNA plays an important role in NB, as it increases proliferation and migration^{99, 100}. The miR-192 has never been tested in SK-N-BE(2) cells, where we found that it is expressed. In conformity with a previous work by Jauhari *et al.*¹⁵⁰, in which miR-192 was also found to be expressed in SH-SY5Y cells, in our study miR-192 was also detected in these cells.

miR-23a acts as oncomiR and, to our knowledge, there are no studies indicating that miR-23a is involved in the TAp73 pathway. Despite this, miR-23a was chosen in this work due to its key role in promoting NB cell migration and invasion^{25, 98}. This miRNA was never tested in SK-N-BE(2) and SH-SY5Y cells. Our results indicated that miR-23a was expressed in both cell lines. Furthermore, a study by Cheng *et al.* revealed that miR-23a was upregulated in metastatic NB tissues when compared to primary NB tissues⁹⁸.

The miRNA let-7 family contains 12 members¹⁵¹ and TAp73 regulates the processing of the tumour suppressor let-7¹⁰⁰. Let-7i overexpression leads to a significant decrease in NB self-renewal and migration¹²⁹. In addition, LIN28B, an inhibitor of let-7i biogenesis, is overexpressed in NB and has been reported to regulate *MYCN* (target of let-7)^{106, 129}. To our knowledge, let-7i has never been tested in SK-N-BE(2) cells, so here we demonstrated that this miRNA is expressed in these cells. In SH-SY5Y cells, we could confirm the expression of let-7i as previously reported¹⁵².

Although some tested miRNAs were expressed in SH-SY5Y and SK-N-BE(2) cells, no significant changes in their levels could be detected after LEM3 treatment. As most of the

miRNAs tested are associated with the p73 pathway, it can also be concluded that the compound does not act via p73-related miRNAs.

Overall, work reports a new activator of TAp73, capable of disrupting TAp73-MDM2 and TAp73-mutp53 interactions. The results also revealed a potent antitumor activity of LEM3 against NB cells, supporting a promising application of this compound in the treatment of aggressive and poor prognosis NB.

6. CONCLUSION

In conclusion, LEM3 revealed an evident antitumour activity, associated with cell cycle arrest and apoptosis, in NB cells harbouring wt or mutp53. Moreover, LEM3 increased the expression levels of TAp73 and subsequent transcriptional activity, decreasing the levels of negative markers of high-risk NB. The results also evidenced the efficacy of the compound in disrupting the interaction of TAp73 with MDM2 and mutp53. Thus, this work reveals the identification of a potential activator of TAp73 and anticancer drug candidate for NB therapy.

Although relevant insights have been provided about the antitumour activity and mode of action of LEM3 in NB, further studies are still required to support the activation of a TAp73-dependent pathway and an *in vivo* efficiency of LEM3 against NB.

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