Targeting neuroblastoma with a new inhibitor of the TAp73 interaction with MDM2 and mutant p53

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Targeting neuroblastoma with a new inhibitor of the TAp73 interaction with MDM2 and mutant p53
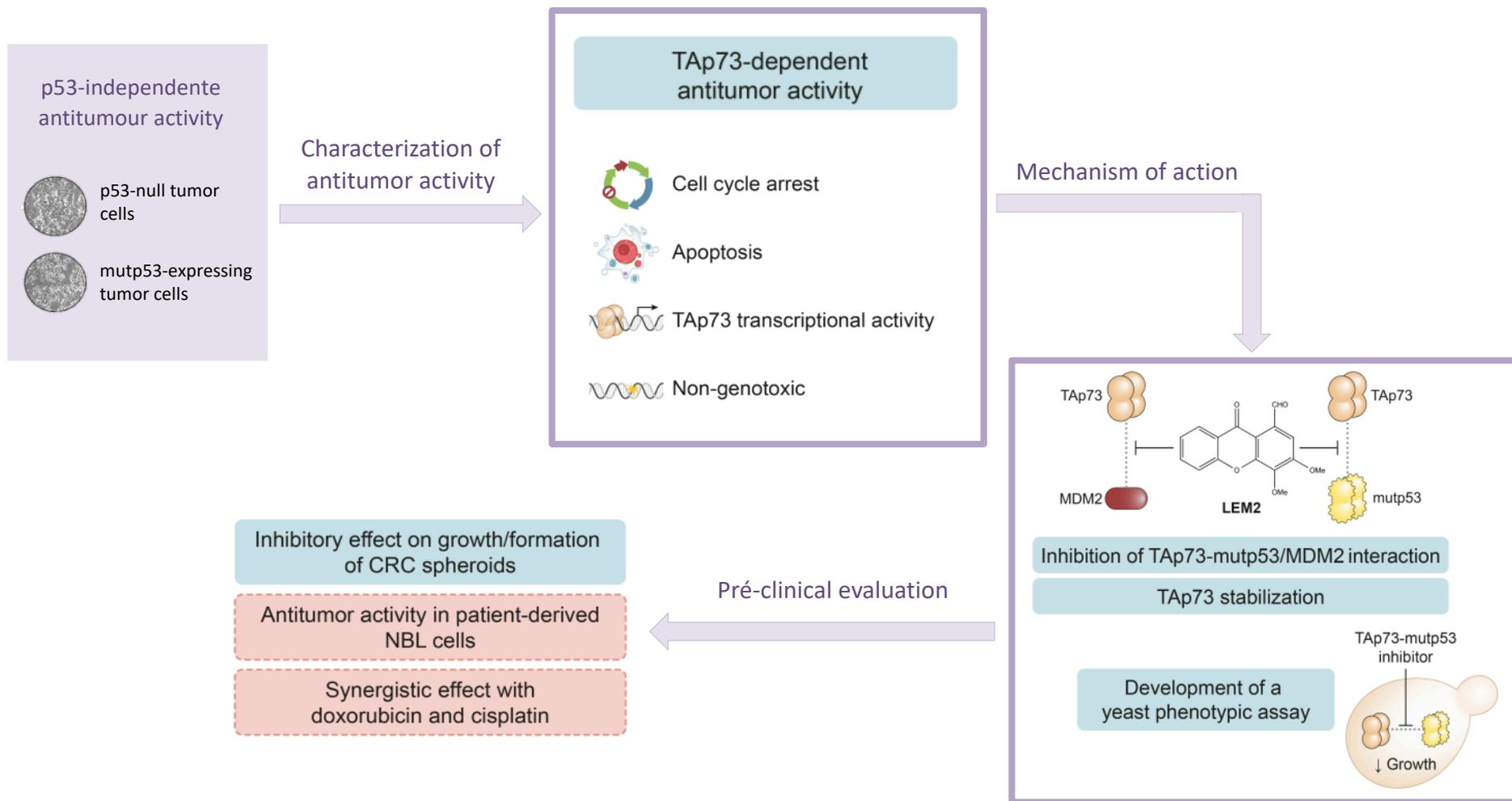
Abstract

TAp73 is a key tumour suppressor protein, regulating the transcription of unique and shared p53 target genes with crucial roles in tumorigenesis and therapeutic response. As such, in tumours with impaired p53 signalling, like neuroblastoma (NBL), TAp73 activation represents an encouraging strategy to suppress tumour growth and chemoresistance.

In this work, we report a new TAp73-activating agent, the 1-carbaldehyde-3,4-dimethoxyxanthone (LEM2), with potent antitumour activity independent of p53 expression. LEM2 was able to release TAp73 from its interaction with both MDM2 and mutant p53 (mutp53), enhancing TAp73 transcriptional activity, cell cycle arrest, and apoptosis in p53-null and mutp53-expressing tumor cells. By cellular thermal shift assay (CETSA), LEM2 induced thermal stabilization of TAp73α but not of MDM2 or mutp53, suggesting the potential interaction of LEM2 with TAp73α. Interestingly, neither LEM2 alcohol 1-(hydroxymethyl)-3,4-dimethoxy-9H-xanthen-9-one (LEMred) or carboxylic acid 3,4-dimethoxy-9-oxo-9H-xanthene-1-carboxylic acid (LEMox) derivatives were able to inhibit the TAp73-MDM2 interaction supporting that LEM2 biological activity was due to the molecule itself and not to its potential derivatives. Consistently with an activation of the TAp73 pathway, LEM2 also displayed potent antitumour activity against patient-derived NBL cells, both alone and in combination of with doxorubicin and cisplatin. Collectively, besides its relevant contribution to the advance of TAp73 pharmacology, LEM2 may pave the way to improved therapeutic alternatives against NBL.

Key words: p73; Carbaldehydic xanthone; Anticancer therapy

The p53 family of tumor suppressor proteins

Stress signals

Hypoxia

Nutrient starvation

DNA damage

Oncogene activation

p53

TAp 73

TAp 63

Targeting p53 family proteins: a valuable strategy in cancer therapy
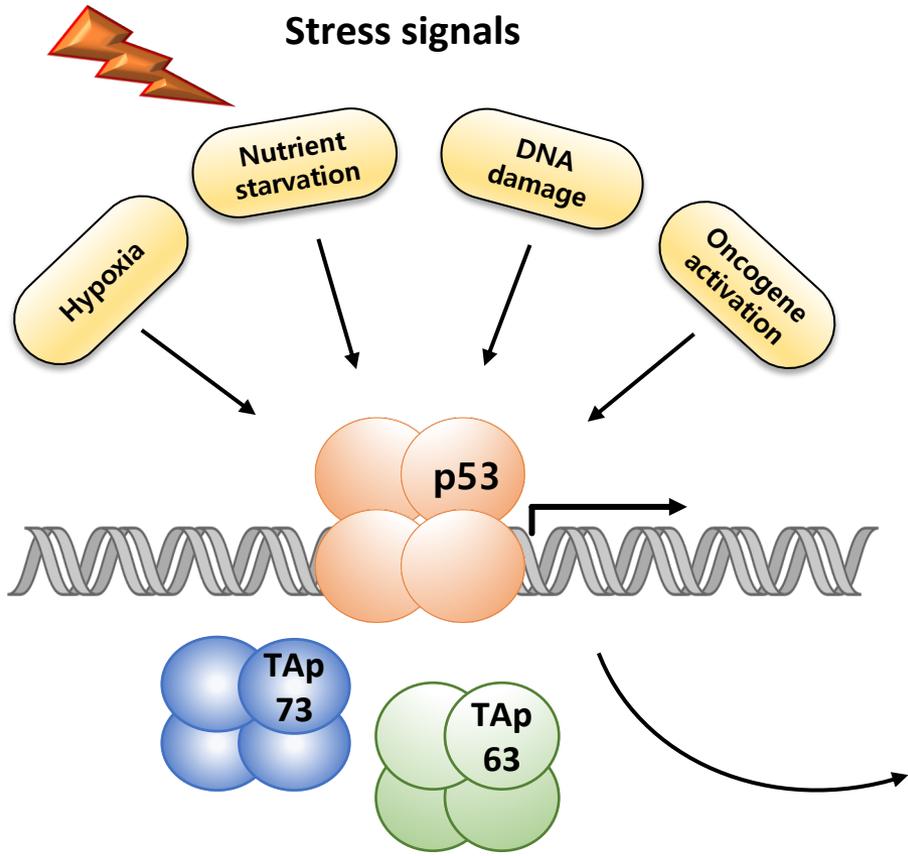
DNA Repair

Cell Cycle Arrest

Senescence

Metabolic Reprogramming

Apoptosis

Tumorigenesis

Crucial role
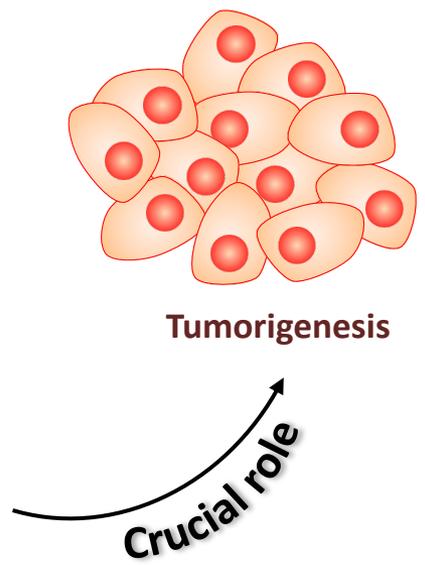
p53 family proteins, p53, p63 and p73 are sequence specific **transcription factors**

Wei and Zaika (2012). J Nucl Acids 2012, 687359.; Kruiswijk F et al., Nat Rev Mol Cell Biol, 2015, 16:393-405.

Targeting TAp73 in Cancer

Anticancer therapeutic strategy
Tumors without p53 or with impaired p53 pathway
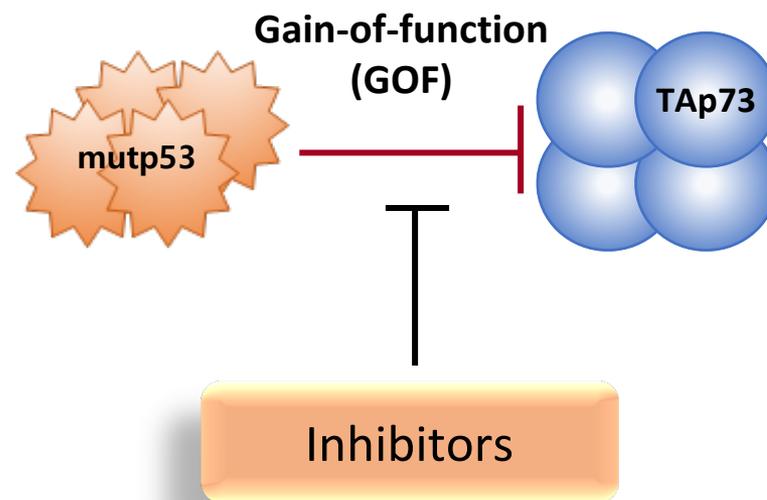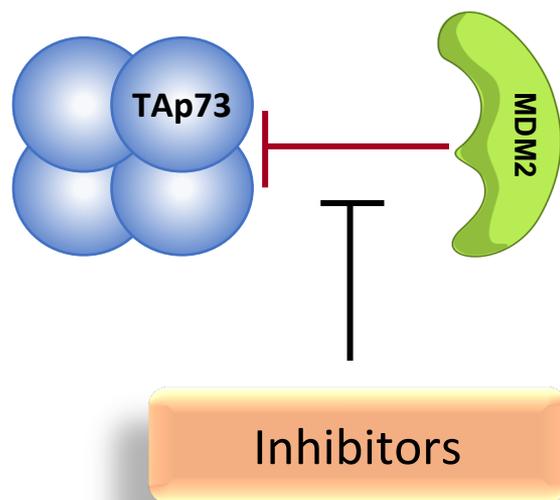
Slade N et al. Curr Pharm Des. 2011;17(6):591-602; Burgess, A et al. Frontiers in Oncology. 2016;6, 7.

Synthetic pathway and reaction conditions to obtain 1-carbaldehyde-3,4-dimethoxyxanthone (LEM2) and putative metabolites LEMred and LEMox
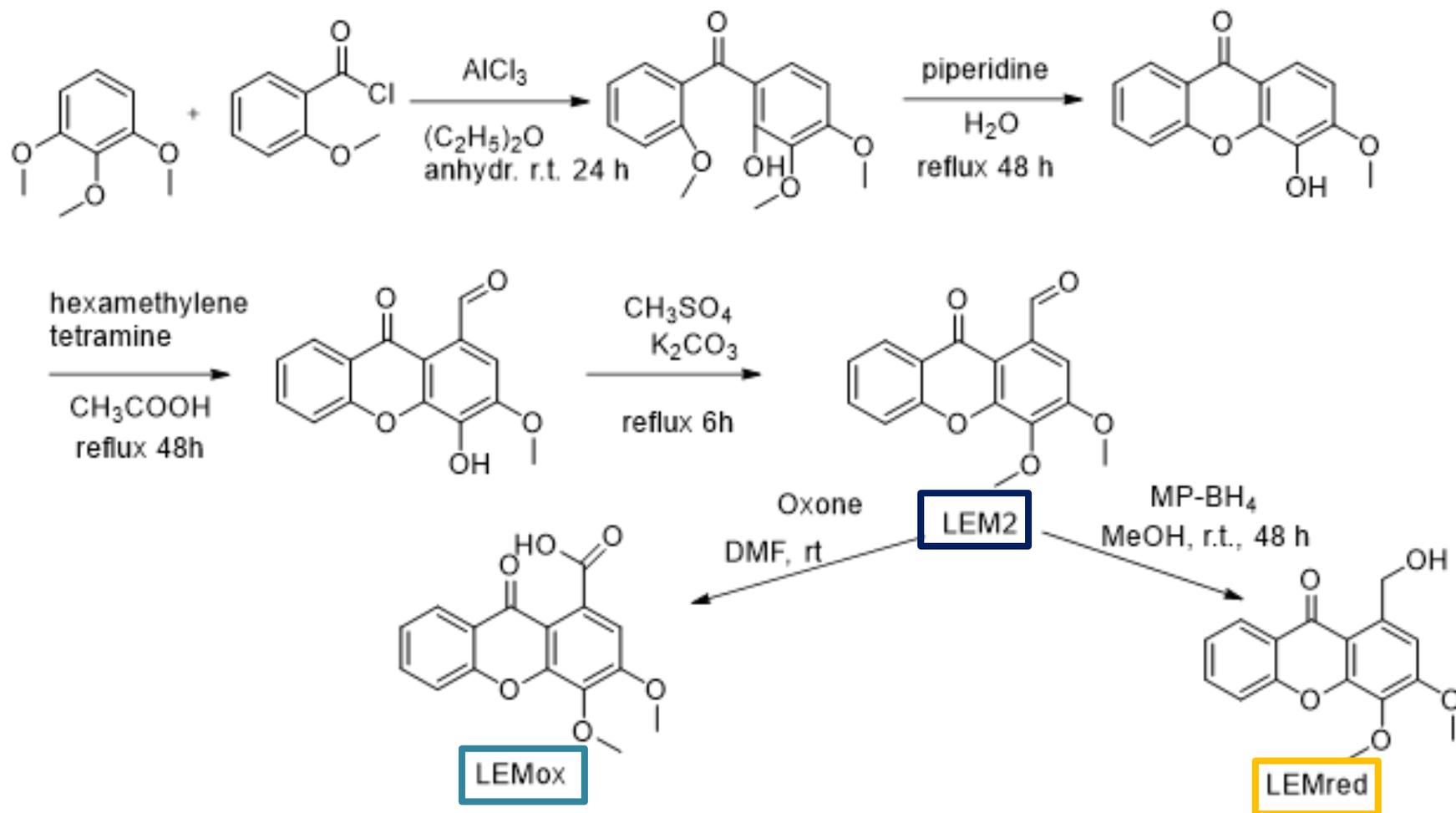
# LEM2 has p53-independent antitumor activity through induction of apoptosis and cell cycle arrest
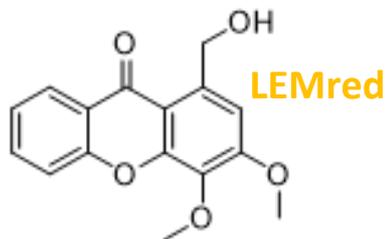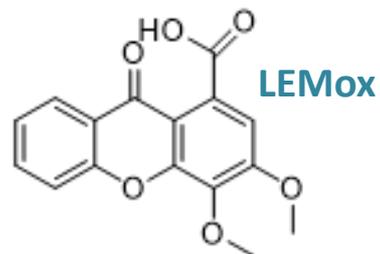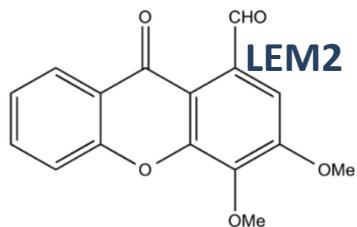
LEM2

LEMox

LEMred

Sulforhodamine B *assay*
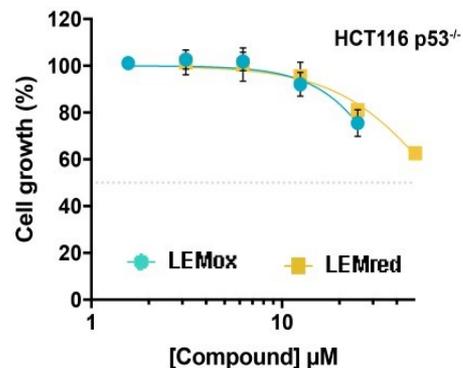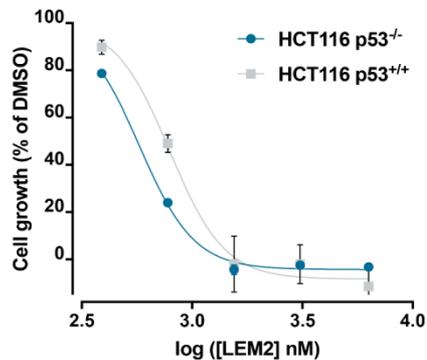
LEM2, LEMox and LEMred were tested for 48h treatment; Data are mean±SEM (n=4).

Cell cycle analysis (PI)
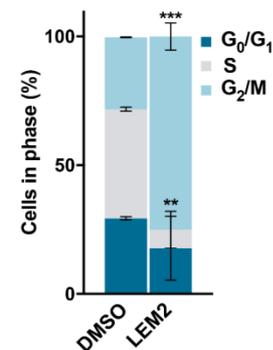
LEM2 was tested at 2µM on HCT116 p53-/- cells for 24h; Data are mean±SEM (n=3); **p<0.01, ***p<0.001.

Apoptosis analysis (Annexin V/PI)
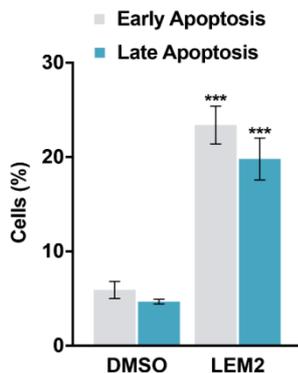
LEM2 was tested at 2µM on HCT116 p53⁻/⁻ cells for 48h treatment. Data are mean±SEM (n=4); ***p<0.001

Western blot
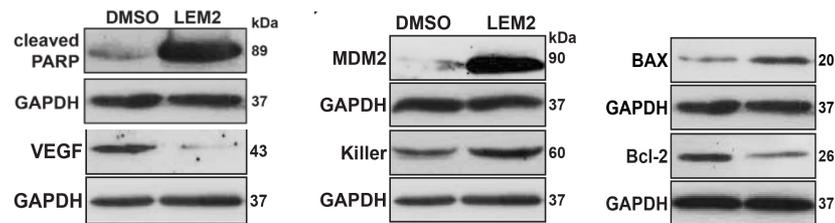
LEM2 was tested at 1µM for 16h or 24h. Immunoblots represent one of three independent experiments.

LEM2 is non-genotoxic and has p53-independent antitumor activity in human cancer cells

3D colon cancer spheroids

Comet assay

Cytokinesis-block micronucleus assay

4-day-old HCT116 p53^{-/-} spheroids, treated with LEM2 for 48h and 72h.
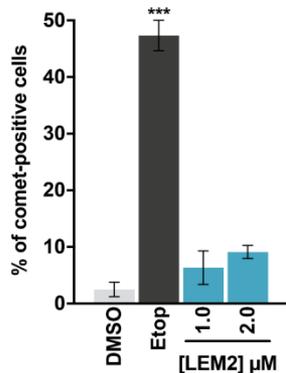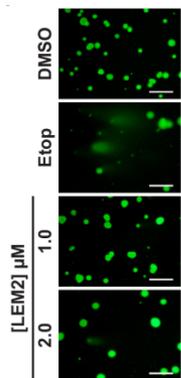
LEM2 was tested on HCT116 p53^{-/-} cells for 48h treatment; Etoposide (Etop; positive control). Representative images (n=3). Quantification of comet-positive cells; one hundred cells were analysed in each group. Data are mean±SEM (n=3); ***p<0.001.
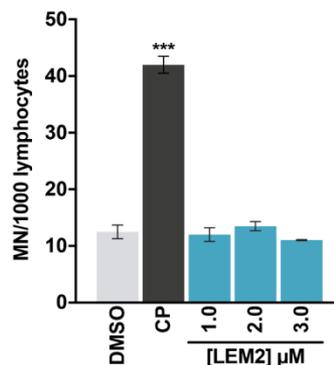
LEM2 was tested on human lymphocytes for 72h; cyclophosphamide (positive control). Data are mean±SEM (n=3); ***p<0.001.
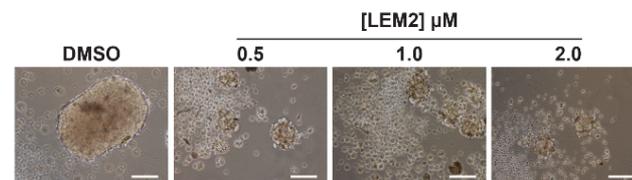

HCT116 p53^{-/-} spheroids formed after 7 days. Spheroids were seeded in the presence of LEM2.

LEM2 activates TAp73 by disruption of its interaction with MDM2 and induction of TAp73 thermal stabilization

Yeast Model
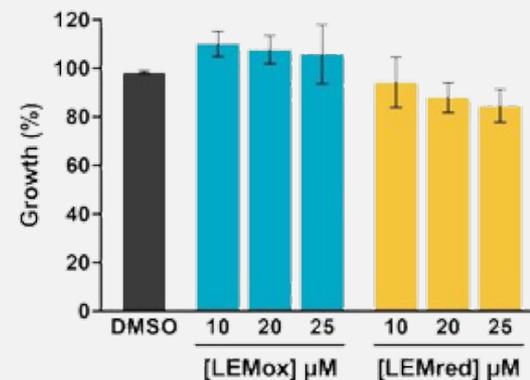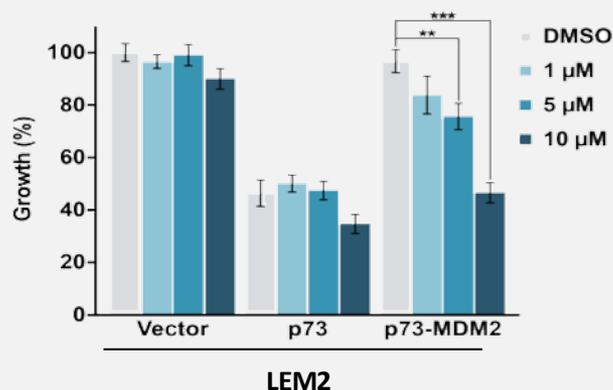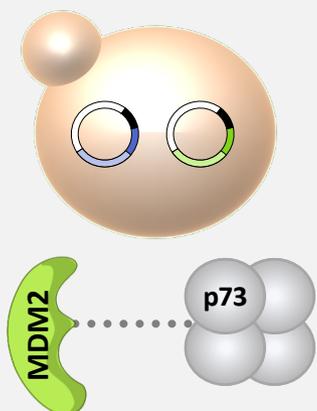
LEM2, LEMrox and LEMred were tested on yeast cells expressing p73 alone co-expressing p73 and MDM2 for 48h. Growth of untreated control yeast was set as 100%; data are mean±SEM (n=4); **p<0.01, ***p<0.001.

Co-immunoprecipitation

CETSA

HCT116p53 ⁻/⁻

HCT116p53 ⁺/⁺

LEM2 was tested at 4 and 5µM on HCT116 p53⁺/⁺ and HCT116 p53⁻/⁻ cells for 24h. Immunoblots represent one of three independent experiments.
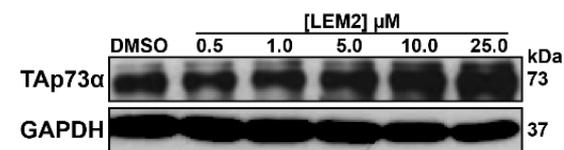
CETSA. HCT116 p53⁻/⁻ cells. Lysates heated at 56°C. (n=3)

LEM2 has potent antitumor activity in mutp53-expressing cancer cells

Sulforhodamine B *assay*
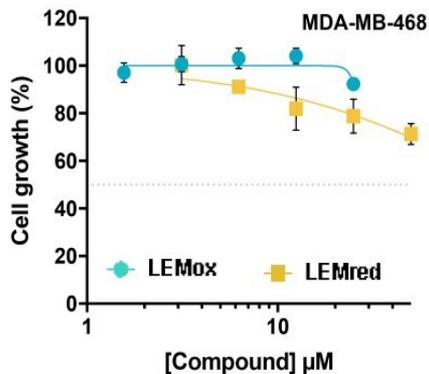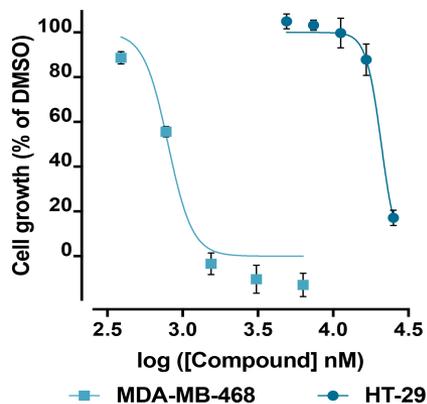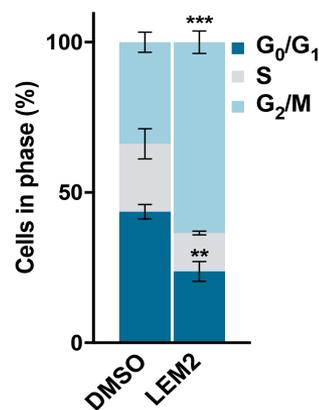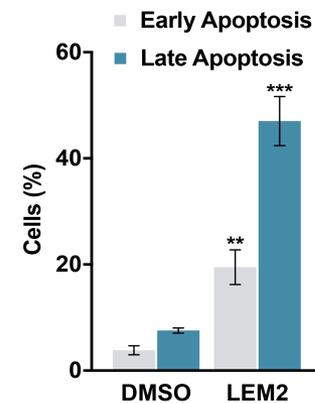

LEM2, LEMox and LEMred were tested at for 48h. Data are mean±SEM (n=4).

Cell cycle analysis (PI)

LEM2 was tested at 1.5µM on MDA-MB-468 for 24h. Data are mean±SEM (n=3); **p<0.01, ***p<0.001.

Apoptosis analysis (Annexin V/PI)

LEM2 was tested at 1.5µM on MDA-MB-468 cells for 48h. Data are mean±SEM (n=4); **p<0.01, ***p<0.001.

p73 protein levels. Immunoblots represent one of three independent experiments.
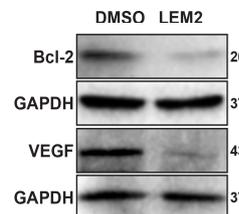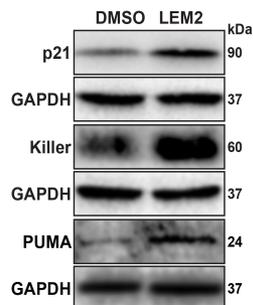
LEM2 was tested at 1µM for 16h or 24h. Immunoblots represent one of three independent experiments.

LEM2 activates TAp73 in mutp53-expressing cancer cells through disruption of the TAp73-mutp53 interaction and TAp73 thermal stabilization

Yeast Model
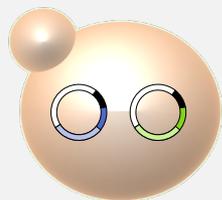

Empty vectors
p73
R273H
p73 + R273H

Yeast growth
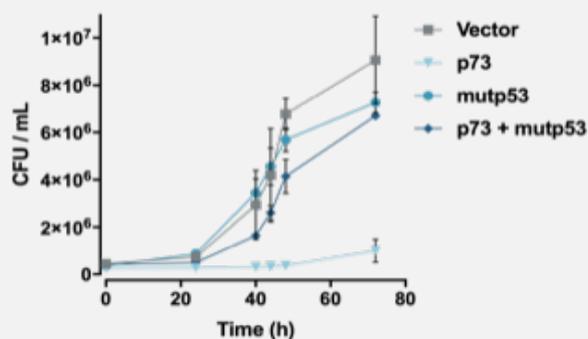
Yeast cells individually expressing p73 or mutp53 R273H, co-expressing p73 and mutp53 R273H, and control yeast were grown for 72h. Data are mean±SEM (n=4).

Yeast screening assay
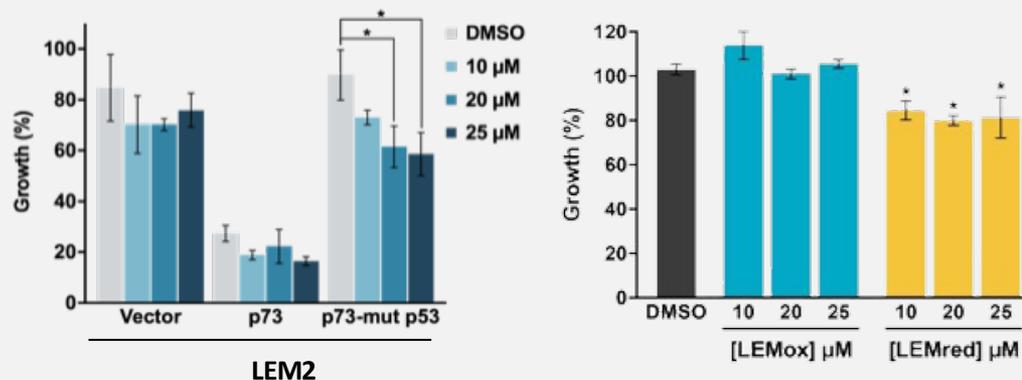
LEM2, LEMox and LEMred were tested on control yeast cells, yeast expressing p73 alone, and yeast co-expressing p73 and mutp53 R273H for 48h. Growth of untreated control yeast was set as 100%. Data are mean±SEM (n=4); *p<0.05.

Co-immunoprecipitation
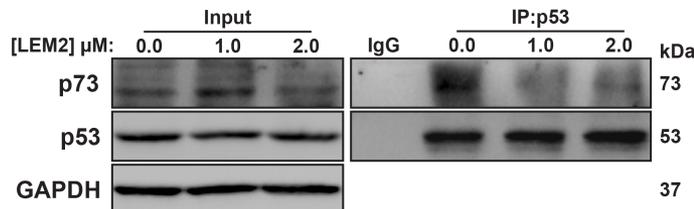
LEM2 was tested at 1 and 2µM on MDA-MB-468 cells for 24h. Immunoblots represent one of three independent experiments.
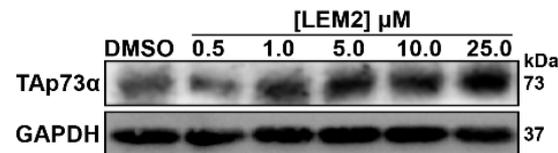
CETSA

CETSA. MDA-MB-468 cells. Lysates heated at 52°C.

Potential application of LEM2 in NBL treatment
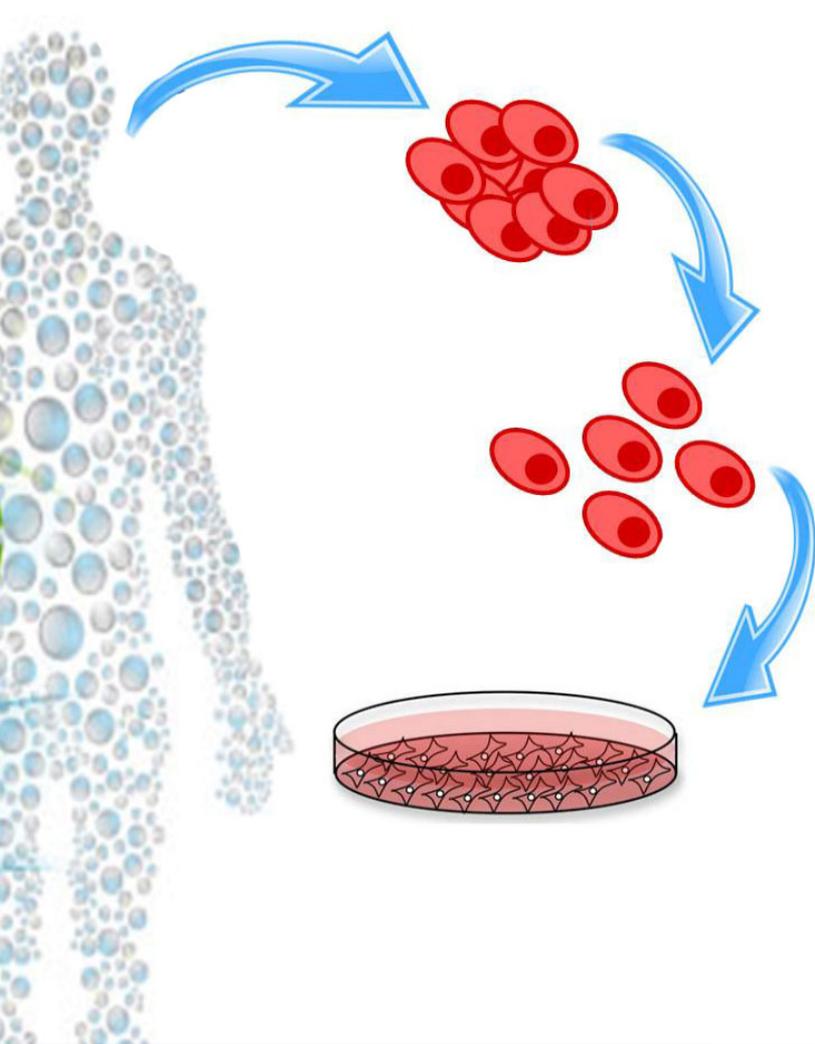
Lack of effective and non toxic strategies, specially in children with metastatic high-risk disease

p73 is found inhibited in NBL cells by interaction with MDM2 and mutant p53

Jennifer Wolter, Paola Angelini & Meredith (2010). Future Oncol. 6(3), 429–444

LEM2 displays potent growth inhibitory effect against NBL cells

Western blot
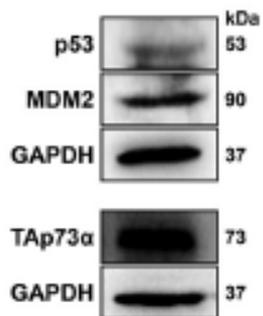
Protein expression analysis by Western blot(n=3).

Sulforhodamine B *assay*

$IC_{50}=0.5\mu M$
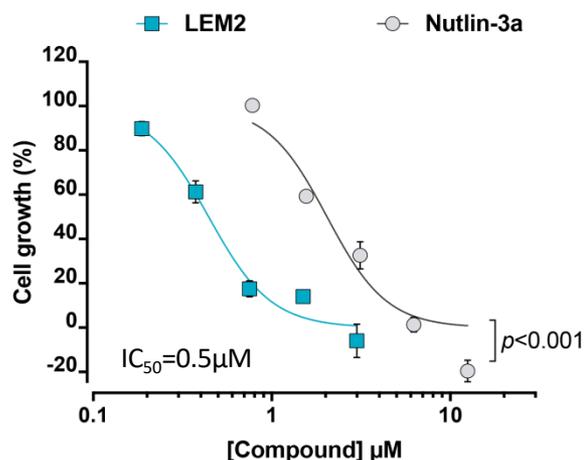
$p<0.001$

IC50 by MTT assay. 0,18-3,00μM LEM2, 0,78-12.5μM Nutlin-3a. 48h treatment. Mean±SEM (n=4). Extra sum-of-squares F test.

Cell cycle analysis (PI)
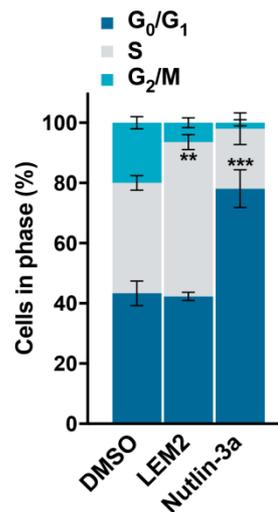
Cell cycle analysis by PI staining. 1μM LEM2, 4μM Nutlin-3a. 48h treatment. Mean±SEM (n=3). **$p<0,01$; ***$p<0,001$ Two-way ANOVA, Dunnet's multiple comparison test

Apoptosis analysis (Annexin V/PI)
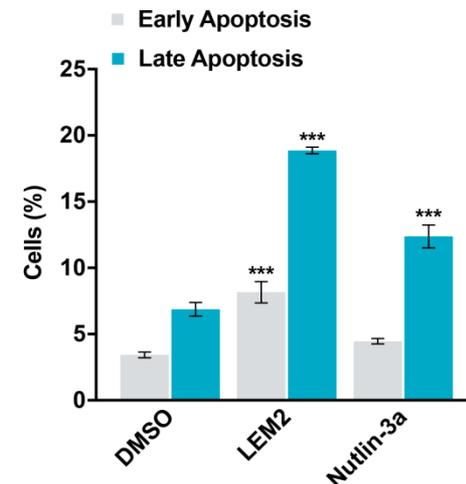
Apoptosis analysis by Anexin V/PI staining. 1μM LEM2, 4μM Nutlin-3ª. 48h de treatment. Mean ± SEM (n=4). ***$p<0,001$ One-way ANOVA, Dunnet's multiple comparison test.

LEM2 was tested at 1 and 2μM on MDA-MB-468 cells for 24h. Immunoblots represent one of three independent experiments.

LEM2 has TAp73-dependent activity in patient-derived NBL cells

Sulforhodamine B *assay*

IC$_{50}$=1.4µM
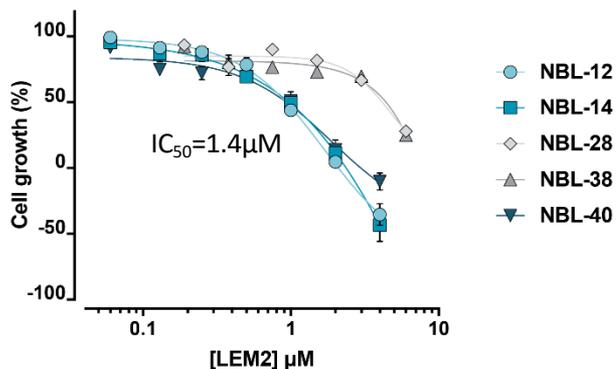
IC50 determined by MTT assay. 0.125-6µM LEM in patient-derived cell lines. 48h treatment. Mean ± SEM (n=5).

Cell cycle analysis (PI)
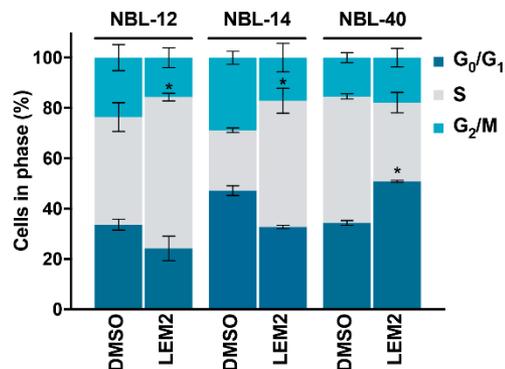
Cell cycle analysis by PI staining. 1.4 µM LEM2. 48h treatment. Mean ± SEM (n=3). *p<0.05. Two-way ANOVA, Dunnet's multiple comparison test

Western blot
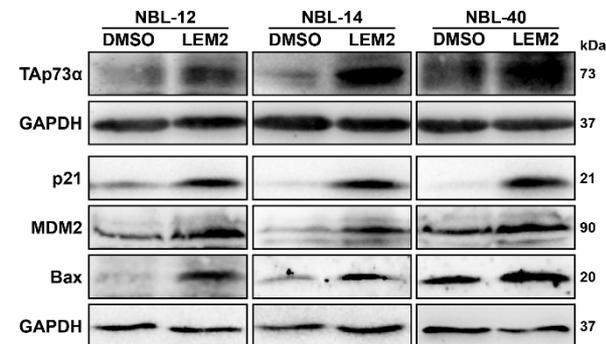
Protein levels by Western blot. 48h treatment. 1.4µM LEM2. (n=3)
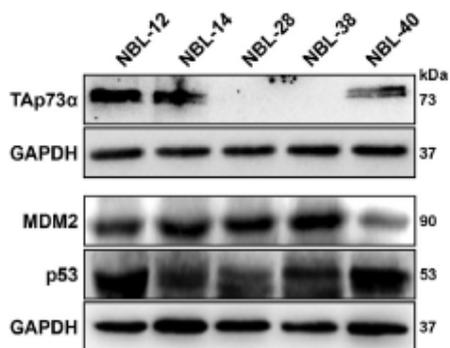
Protein analysis by Western bklot in patient-derived cell lines. (n=3)

RT-qPCR

CDKN1A

MDM2

BAX
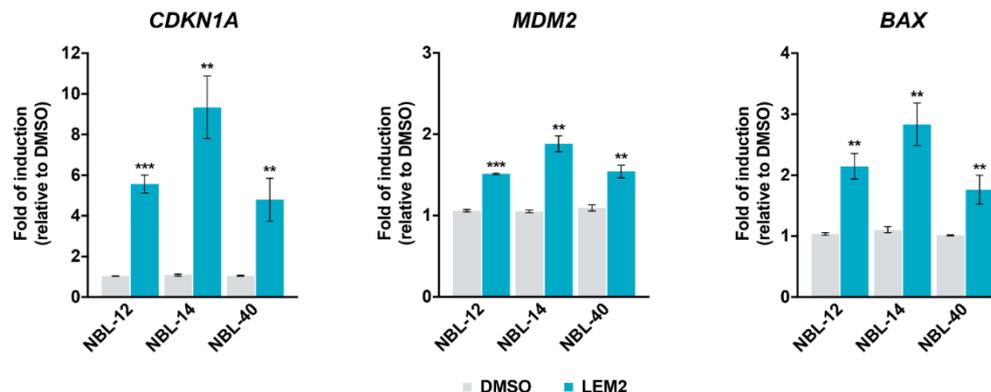
mRNA levels analysis by RT-qPCR. 1.4µM LEM2. 24h treatment. Fold of induction relative to solvent. Mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001. Two-way ANOVA, Dunnet's multiple comparison test.

LEM2 sensitizes immortalized and patient-derived NBL cells to doxorubicin and cisplatin
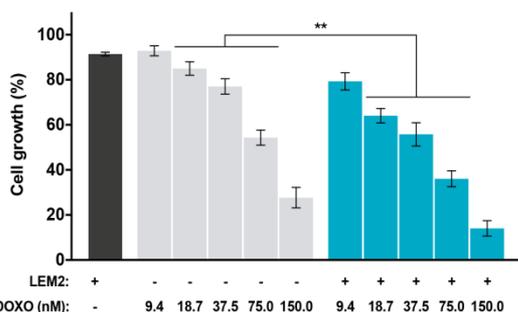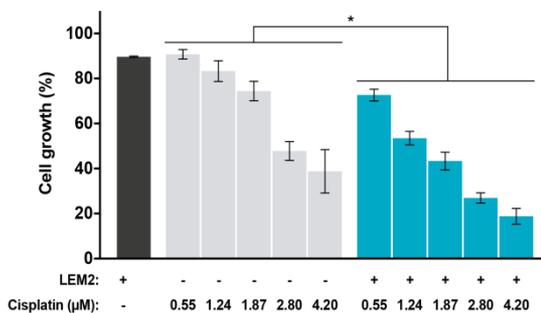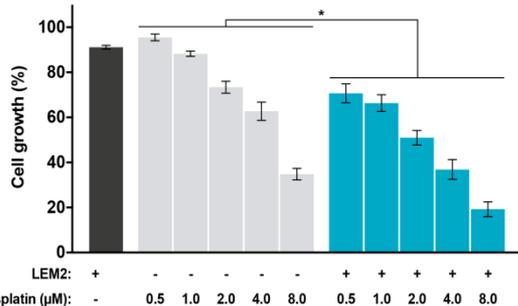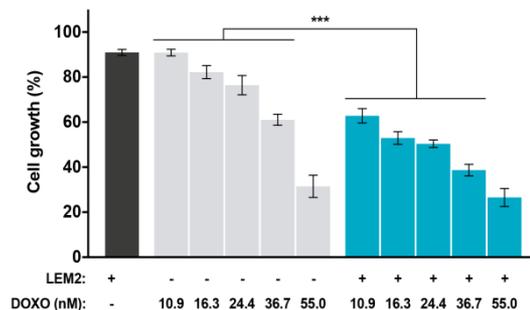
Determined by MTT assay. Increasing doses of doxorubicin/cisplatin, 0.21μM (SH-SY5Y) or 0.48μM (patient-derived cells) LEM2. 48h treatment. Mean ± SEM (n=5). *p<0.05, **p<0.01, ***p<0.001. Two-way ANOVA, Sidak's multiple comparison test.

Drug combination with LEM2	Mutually nonexclusive CI		Dose reduction index (DRI)
	CI	Profile	
SH-SY5Y cells			
DOXO (nM)			
9.37	1.007	Additive	2.911
18.7	0.859	Synergy	2.688
37.5	0.987	Synergy	1.776
75	0.896	Synergy	1.696
150	0.619	Synergy	2.310
Cisplatin (μM)			
0.5	0.803	Synergy	5.120
1	0.899	Synergy	3.003
2	0.860	Synergy	2.470
4	0.889	Synergy	1.948
8	0.787	Synergy	1.949
Patient-derived NBL-14 cells			
DOXO (nM)			
10.9	0.655	Synergy	3.101
16.3	0.665	Synergy	2.459
24.4	0.827	Synergy	1.713
36.7	0.901	Synergy	1.389
55	0.984	Synergy	1.168
Cisplatin (μM)			
0.55	0.938	Synergy	3.557
1.24	0.889	Synergy	2.561
1.87	0.892	Synergy	2.177
2.8	0.780	Synergy	2.256
4.2	0.787	Synergy	1.998

Evaluated using CompuSyn software. CI<1 synergy, 1<CI<1.1 additive effect, CI>1.1 antagonism. (n=5)

Conclusions

- LEM2 displays p53-independent antitumor activity in both p53-null and mutp53- expressing cells.

- LEM2 growth inhibitory effect is TAp73-dependent, and associated with cell cycle arrest, apoptosis, and induction of TAp73 target genes, but not with genotoxicity.

- LEM2 is an activator of TAp73 through disruption of its interaction with MDM2 and mutp53, and TAp73 stabilization.

- The potential application of LEM2 for NBL treatment was evidenced.

TAp73 activation
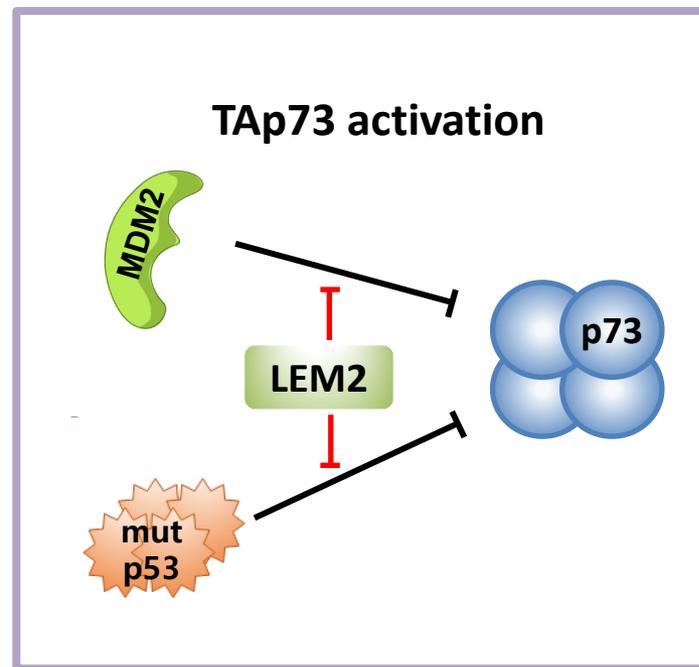
LEM2 may represent a **promising anticancer drug candidate**, particularly **against p53-impaired tumors**

Acknowledgments

5th International Electronic Conference on Medicinal Chemistry
1-30 November 2019

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