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

Liliana Raimundo¹, Sara Gomes¹, Joana Soares¹, Joana B. Loureiro¹, Mariana Leão¹, Helena Ramos¹, Madalena N. Monteiro¹, Juliana Calheiros¹, Nair Nazareth¹, Joana Almeida¹, Agostinho Lemos², Joana Moreira², Madalena Pinto², Petr Chlapek³⁴, Renata Veselsk³⁴, Emília Sousa², Lucília Saraiva¹

¹LAQV/REQUIMTE, Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal. ²CIIMAR, Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal. ³Laboratory of Tumor Biology, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic. ⁴International Clinical Research Center, St. Anne’s University Hospital, Brno, Czech Republic.

*lucilia.saraiva@ff.up.pt
*esousa@ff.up.pt
*liliana-raimundo@live.com
Targeting neuroblastoma with a new inhibitor of the TAp73 interaction with MDM2 and mutant p53

Characterization of antitumor activity

- Cell cycle arrest
- Apoptosis
- TAp73 transcriptional activity
- Non-genotoxic

p53-independent antitumour activity

- p53-null tumor cells
- mutp53-expressing tumor cells

Mechanism of action

- Inhibition of TAp73-mutp53/MDM2 interaction

Pré-clinical evaluation

- Inhibitory effect on growth/formation of CRC spheroids
- Antitumor activity in patient-derived NBL cells
- Synergistic effect with doxorubicin and cisplatin

TAp73 stabilization

Development of a yeast phenotypic assay

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

sponsors: MDPI pharmaceuticals
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; Carbaldeydic xanthone; Anticancer therapy
The p53 family of tumor suppressor proteins

Stress signals
- Hypoxia
- Nutrient starvation
- DNA damage
- Oncogene activation

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

Gain-of-function (GOF)
mutp53

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

**Sulforhodamine B assay**

Cell growth (%) of DMSO

- HCT116 p53⁻/⁻
- HCT116 p53⁺/+;

**Cell cycle analysis (PI)**

Cells in phase (%)

- DMSO
- LEM2

**Apoptosis analysis (Annexin V/PI)**

Early Apoptosis

Late Apoptosis

**Western blot**

- cleaved PARP
- GAPDH
- VEGF
- GAPDH

**5th International Electronic Conference on Medicinal Chemistry**

1-30 November 2019

---

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

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

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

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

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

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

**Comet assay**

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.

**Cytokinesis-block micronucleus assay**

LEM2 was tested on human lymphocytes for 72h; cyclophosphamide (positive control). Data are mean±SEM (n=3); ***p<0.001.

**3D colon cancer spheroids**

4-day-old HCT116 p53\(^{-/-}\) spheroids, treated with LEM2 for 48h and 72h.

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 was tested at 4 and 5µM on HCT116 p53+/+ and HCT116 p53/- cells for 24h. Immunoblots represent one of three independent experiments.

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.

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

Cell cycle analysis (PI)

Apoptosis analysis (Annexin V/PI)

Sulfonhodamine B assay

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

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.

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

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 growth

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

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

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

Lack of effective and non toxic strategies, specially in children with metastatic high-risk disease

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**

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 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

IC50 determined by MTT assay. 0.125-6µM LEM in patient-derived cell lines. 48h treatment. Mean ± SEM (n=5).

Cell cycle analysis (PI)

IC50 = 1.4µM

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 blot in patient-derived cell lines. (n=3)

RT-qPCR

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.

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.

LEM2 may represent a promising anticancer drug candidate, particularly against p53-impaired tumors.
Acknowledgments

This work received financial support from PT national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through grant UID/QUI/50006/2019. This work received financial support from the European Union (FEDER funds through the Operational Competitiveness Program (COMPETE) POCI-01-0145-FEDER-006684/POCI-01-0145-FEDER-007440 and (3599-PPCDT) PTDC/DTP-FTO/1981/2014 – POCI-01-0145-FEDER-016581) and the FCT grants PTDC/QUIQOR/29664/2017, POCI-01-0145-FEDER-028736 (PTDC/SAU-PUB/28736/2017). We thank FCT and ESF (European Social Fund) through POCH (Programa Operacional Capital Humano) for: L. Raimundo PhD grant ref. SFRH/BD/117949/2016; J. Loureiro PhD grant ref SFRH/BD/128673/2017; H. Ramos PhD grant ref SFRH/BD/119144/2016. J. Calheiro thanks ICETA for her grant ref. ICETA2019-71. M. Monteiro thanks ICETA for her grant ref. ICETA2019-70 We thank (POCH), specifically the BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences; PD/00016/2012).