A tryptophanol-derived oxazolopiperidone lactam is cytotoxic against tumors via inhibition of p53 interaction with murine double minute proteins

Joana Soares^{a,b,1}, Liliana Raimundo^{a,b,1}, Nuno A. L. Pereira^d, Daniel J. V. A. dos Santos^e, Maria Pérez^f, Glória Queiroz^{a,c}, Mariana Leão^{a,b}, Maria M. M. Santos^{d,*} and Lucília Saraiva^{a,b,*}

^aUCIBIO/REQUIMTE, ^bLaboratório de Microbiologia, Departamento de Ciências Biológicas, ^cLaboratório de Farmacologia, Departamento de Ciências do Medicamento, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 164, 4050-313, Porto, Portugal; ^dResearch Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, University of Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal; ^eREQUIMTE, Departamento de Química e Bioquímica, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal; ^fLaboratory of Organic Chemistry, Faculty of Pharmacy and Institute of Biomedicine, University of Barcelona, Av. Joan XXIII, s/n, 08028 Barcelona, Spain.

¹Authors contributed equally to this work

*Corresponding authors:

Lucília Saraiva, REQUIMTE, Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 164, 4050-313, Porto, Portugal, Tel.: +351 220428584; fax: +351 226093390, lucilia.saraiva@ff.up.pt

25 Maria M. M. Santos, Research Institute for Medicines (iMed.Ulisboa), Faculty of
26 Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal,
27 Tel:+351 217946451, Fax: +351 217946470, mariasantos@ff.ulisboa.pt

28 **Abbreviations**

29 $\Delta\psi_m$ - mitochondrial membrane potential

30 CFU - colony-forming units

31 co-IP - co-immunoprecipitation

32 cyt *c* - cytochrome *c*

33 DMSO - dimethyl sulfoxide

34 FCCP - p-trifluoromethoxyphenylhydrazone

35 IgG - immunoglobulin G

36 PI - propidium iodide

37 MDM2 - murine double minute 2

38 MDMX - murine double minute X

39 MMP - mitochondrial membrane permeabilization

40 ROS - reactive oxygen species

41 SRB - sulforhodamine B

42 wt – wild-type

43

44 **Chemical compounds studied in this article**

45 Nutlin-3a (PubChem CID: 11433190); SJ-172550 (PubChem CID: 1317464);

46 Doxorubicin (PubChem CID: 443939); Etoposide (PubChem CID: 36462)

Abstract

Inactivation of the p53 tumor suppressor protein by interaction with murine double minute (MDM) proteins, MDM2 and MDMX, is a common event in human tumors expressing wild-type p53. In these tumors, the simultaneous inhibition of these interactions with MDMs, for a full p53 reactivation, represents a promising anticancer strategy. Herein, we report the identification of a dual inhibitor of the p53 interaction with MDM2 and MDMX, the (*S*)-tryptophanol OXAZ-1, from the screening of a small library of enantiopure tryptophanol-derived oxazolopiperidone lactams, using a yeast-based assay. With human colon adenocarcinoma HCT116 cell lines expressing wild-type p53 (HCT116 p53^{+/+}) and its p53-null isogenic derivative (HCT116 p53^{-/-}), it was shown that OXAZ-1 induced a p53-dependent tumor growth-inhibitory effect. In fact, OXAZ-1 induced p53 stabilization, up-regulated p53 transcription targets, such as MDM2, MDMX, p21, Puma and Bax, and led to PARP cleavage, in p53^{+/+}, but not in p53^{-/-}, HCT116 cells. In addition, similar tumor cytotoxic effects were observed for OXAZ-1 against MDMX-overexpressing breast adenocarcinoma MCF-7 tumor cells, commonly described as highly resistant to MDM2-only inhibitors. In HCT116 p53^{+/+} cells, the disruption of the p53 interaction with MDMs by OXAZ-1 was further confirmed by co-immunoprecipitation. It was also shown that OXAZ-1 potently triggered a p53-dependent mitochondria-mediated apoptosis, characterized by reactive oxygen species generation, mitochondrial membrane potential dissipation, Bax translocation to mitochondria, and cytochrome *c* release, and exhibited a p53-dependent synergistic effect with conventional chemotherapeutic drugs.

Collectively, in this work, a novel selective activator of the p53 pathway is reported with promising antitumor properties to be explored either alone or combined

72 with conventional chemotherapeutic drugs. Moreover, OXAZ-1 may represent a
73 promising starting scaffold to search for new dual inhibitors of the p53-MDMs
74 interaction.

75

76 **Keywords:** p53; murine double minute proteins; p53-MDMs interaction;
77 oxazolopiperidone lactam; antitumor activity; mitochondria-mediated apoptosis

1. Introduction

The p53 transcription factor is a major regulator of several cellular processes involved in tumor progression, such as cell cycle arrest and apoptosis [1,2]. The inactivation of p53 oncosuppressor function is a commonly event in human cancers either by mutation in the *TP53* gene [1], or by interaction of the p53 protein with endogenous inhibitors [2]. In fact, in tumors retaining a wild-type (wt) p53 status, the activity of this protein is suppressed due to interaction with two structurally related negative regulators, murine double minute (MDM) proteins, MDM2 and MDMX. Disruption of the p53-MDMs regulatory network has therefore clear implications in tumorigenesis and presents exciting opportunities for cancer therapy [2]. However, to date, most of the current efforts have been focused on the p53-MDM2 interaction [2]. The role of MDMX in the fine regulation of p53 is still emerging, but it is well-established that even normal levels of MDMX can partially silence activated p53. Additionally, MDMX overexpression (namely in retinoblastomas and melanomas) renders cancer cells highly resistant to MDM2-only inhibitors, such as nutlin-3a, since they are unable to liberate p53 from MDMX [3]. This clearly indicates that dual inhibition of the p53 interaction with MDM2 and MDMX may substantially improve the outcome of this p53 activation strategy due to the full p53 activation [2]. In spite of this, to date, just few small molecule dual inhibitors of the p53-MDMs interaction have been identified, namely a pyrrolopyrimidine [4], an indolyl-hydantoin (RO-5963;[3]) and a cis-imidazoline derivative (H109; [5]).

Recently, our group developed a new approach, based on yeast cells co-expressing human p53 and MDM2 or MDMX, for the targeted screening of inhibitors of the p53 interaction with MDM2 [6] and MDMX [7]. The efficacy of these cell systems for the screening of p53-MDMs interaction inhibitors was demonstrated by the

identification of two new small molecule inhibitors of the p53-MDM2 interaction, a pyranoxanthone [7] and an oxazoloisoindolinone [8].

In this work, we report the identification of a new small molecule dual inhibitor of the p53-MDMs interaction, the (*S*)-tryptophanol derivative OXAZ-1 (Fig. 1), from the screening of a small library of enantiopure tryptophanol-derived oxazolopiperidone lactams using a yeast-based approach. OXAZ-1 exhibits a p53-dependent *in vitro* tumor cytotoxic effect via p53 activation with dual inhibition of the p53 interaction with MDM2 and MDMX. The promising antitumor activity of OXAZ-1 is reinforced by its ability to trigger a p53-dependent mitochondria-mediated apoptosis, as well as by its p53-dependent synergistic effect with conventional chemotherapeutic drugs.

1.5 column

Figure 1. Chemical structure of tryptophanol-derived oxazolopiperidone lactams evaluated in yeast.

2. Materials and Methods

2.1. Compounds

OXAZ-2, OXAZ-3, OXAZ-4, OXAZ-5, and OXAZ-6, whose molecular structures are shown in Figure 1, were synthesized according to the described procedures [9-11]. Nutlin-3a was from Alexis Biochemicals (Grupo Taper, Sintra, Portugal); doxorubicin and SJ-172550 were from Sigma-Aldrich (Sintra, Portugal); etoposide was from Calbiochem (VWR, Carnaxide, Portugal). All tested compounds were dissolved in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Sintra, Portugal).

2.2. Synthesis of OXAZ-1

A solution of (*S*)-tryptophanol-derived oxazolopiperidone lactam OXAZ-5 (0.2 g, 0.548 mmol) in CH₂Cl₂ (10 mL) was cooled to 0°C. *p*-Toluenesulphonyl chloride (0.133 g, 0.701 mmol) and tetrabutylammonium chloride (cat., 10% mmol) were added and the mixture was stirred for 10 min. Then, an aqueous solution of NaOH (30% m/v, 0.5 v CH₂Cl₂) was added and the reaction was allowed to stand at room temperature for 24 h. After this period, the reaction was diluted with CH₂Cl₂ (10 mL) and the phases were separated. The organic phase was washed with HCl (1 M, 10 mL) and dried over Na₂SO₄. After solvent evaporation the crude compound was purified by flash chromatography (EtOAc/*n*-hexane 1:1). The desired compound was obtained as a white crystal solid after recrystallization from dichloromethane. ¹H NMR (400 MHz, CDCl₃) δ 7.96 (d, *J* = 8.1 Hz, 1H), 7.72 (m, 3H), 7.36 (s, 1H), 7.31 (t, *J* = 7.7 Hz, 1H), 7.25 (m, 1H), 7.21 (m, 2H), 4.70 (dd, *J* = 9.9, 2.5 Hz, 1H), 4.23 (m, 1H), 3.95 (d, *J* = 9.4 Hz, 1H), 3.74 (t, *J* = 7.8 Hz, 1H), 3.70 (s, 3H), 3.58 (d, *J* = 14.1 Hz, 1H), 2.68 (dd, *J* = 14.0, 9.8 Hz, 1H), 2.60 (dd, *J* = 17.6, 4.6 Hz, 1H), 2.37 (m, 3H), 2.33 (s, 3H), 2.28 (d, *J* = 12.0 Hz, 1H), 2.10 (dd, *J* = 17.6, 10.2 Hz, 1H), 1.21 (m, 1H). Elemental Anal. calcd. for

C₂₆H₂₈N₂O₆S: C 62.89, H 5.68, N 5.64, found: C 62.98, H 5.84, N 6.00. The ¹H NMR spectra was found to be identical to the one described in [11].

2.3. Yeast targeted screening assay

Saccharomyces cerevisiae (strain CG379) expressing human wt p53 alone and combined with human MDM2 or MDMX were obtained in previous works [6,7]. For expression of human proteins (routinely grown in minimal selective medium), cells were diluted to 0.05 OD₆₀₀ in selective induction medium containing 2% (w/w) galactose, 1% (w/w) raffinose, 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 µg/mL) except leucine and tryptophan. Yeast cells were incubated at 30 °C under continuous orbital shaking (200 r.p.m.) with 0.1 – 50 µM compounds or 0.1% DMSO only, for approximately 42 h (time required by control yeast, co-transformed with the empty vectors and incubated with DMSO only, to achieve 0.4 OD₆₀₀). Yeast growth was analyzed by counting the number of colony-forming units (CFU) after 2 days incubation at 30 °C on Sabouraud Dextrose Agar from Liofilchem (Frilabo, Porto, Portugal).

2.4. Human tumor cell lines and growth conditions

The human colon adenocarcinoma HCT116 cell line expressing wt p53 (HCT116 p53^{+/+}) and its full-length p53-null isogenic derivative (HCT116 p53^{-/-}), and MCF-7 (human breast adenocarcinoma, EACC) were kindly provided by Alberto Inga (CIBIO, University of Trento, Italy). Cell lines were routinely cultured in RPMI-1640 with ultraglutamine medium from Lonza (VWR, Carnaxide, Portugal) supplemented

with 10% fetal bovine serum from Gibco (Alfagene, Carcavelos, Portugal) and maintained in a humidified incubator at 37 °C with 5% CO₂ in air.

2.5. Sulforhodamine B (SRB) assay

For the analysis of the effect of OXAZ-1 on the *in vitro* growth of human tumor cell lines, cells were plated in 96-well plates at a final density of 5.0×10^3 cells/well and incubated for 24 h. Cells were then exposed to serial dilutions of the compound (from 1.85 to 150 µM). The effect of OXAZ-1 on cellular integrity was analyzed following 48 h incubation, using the SRB assay. Briefly, following fixation with 10% trichloroacetic acid, plates were stained with 0.4% SRB, both from Sigma-Aldrich (Sintra, Portugal), and washed with 1% acetic acid. The bound dye was then solubilized with 10 mM Tris Base and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc., Synergy MX, USA). The solvent of the compound (DMSO) corresponding to the maximum concentration used in these assay (0.25%) was included as control. The concentration of compound OXAZ-1 that causes a 50% reduction in the net protein increase in cells during treatment (GI₅₀, growth inhibition of 50%) was thereafter determined.

For combined treatments, cells were treated with the GI₁₀ concentration of OXAZ-1 (or DMSO only) and with increasing concentrations of doxorubicin (9.38 to 150 nM) or etoposide (0.38 to 6.00 µM). The effect of combined treatments on cell growth was analyzed after 48 h incubation, using the SRB assay. To further quantify the synergistic effect of the combined treatment, the following equation was used, basically as reported [12]: $Q = E_a + b / (E_a + E_b - E_a \times E_b)$, where Q is the combination index of the combined treatment, $E_a + b$ represents the cell proliferative inhibition rate of combined drugs, E_a and E_b are signs of the cell proliferative inhibition rate of each

192 drug. After calculation: $Q < 0.85$, $Q > 1.15$ and $0.85 < Q < 1.15$ indicate antagonism,
193 synergy, and additive effect, respectively.

195 **2.6. Analysis of cell cycle and apoptosis in human tumor cell lines**

196 HCT116 and MCF-7 cells were plated in 6-well plates at a final density of $1.5 \times$
197 10^5 cells/well. After 24 h incubation, cells were treated with the GI_{50} concentration of
198 OXAZ-1 or DMSO only for 24 h. For cell cycle analysis, cells were thereafter fixed in
199 ice-cold 70% ethanol and incubated at 37 °C with RNase A from Sigma-Aldrich (Sintra,
200 Portugal) at a final concentration of 20 μ g/mL for 15 min, and further incubated with 50
201 μ g/mL propidium iodide (PI) from Fluka (Sigma-Aldrich, Sintra, Portugal) for 30 min,
202 followed by flow cytometric analysis. For apoptosis analysis, cells were analyzed by
203 flow cytometry using the Annexin V-FITC Apoptosis Detection Kit I from BD
204 Biosciences (Enzifarma, Porto, Portugal), according to the manufacturer's instructions.
205 Synergistic effects of combined treatments, between OXAZ-1 (or DMSO only) at GI_{10}
206 concentration and 9.38 nM doxorubicin or 0.38 μ M etoposide, on apoptosis was
207 analyzed as described in section 2.5.

209 **2.7. Western blot analysis**

210 Whole protein extracts from yeast and human cell lines were obtained as
211 described [7,8]. For mitochondrial and cytosolic fractions of human tumor cell lines, the
212 Mitochondrial Fractionation Kit from Active Motif (Frilabo, Porto, Portugal) was used
213 according to the manufacturer's instructions. Protein extracts were quantified using the
214 Coomassie staining Bradford from Sigma-Aldrich (Sintra, Portugal). Proteins (40 μ g)
215 were electrophoresed using a 10% SDS-PAGE and transferred to a Whatman
216 nitrocellulose membrane from Protan (VWR, Carnaxide, Portugal). Membranes were

blocked with 5% milk and probed with a mouse monoclonal anti-p53 (DO-1), anti-MDM2 (D-12), anti-Bax (2D2), anti-PUMA (B-6), anti-PARP (C2-10), and anti-cytochrome *c* (A-8), or a rabbit polyclonal anti-p21 (C-19), anti-MDMX (A300), and anti-actin (C11), followed by an anti-mouse or anti-rabbit horseradish-peroxidase (HRP)-conjugated secondary antibody. For loading control, membranes were stripped and reprobed with a mouse monoclonal anti-yeast phosphoglycerate kinase (Pgk1p) or anti-GAPDH (6C5). For analysis of mitochondrial and cytosolic fractions, membranes were reprobed with the loading controls mouse monoclonal anti-GAPDH (6C5) or anti-COX IV (F-8), used to exclude putative contamination of cytosolic and mitochondrial fractions, respectively. All antibodies were purchased from Santa Cruz Biotechnology (Frlabo, Porto, Portugal), except the anti-MDMX obtained from Bethyl Laboratories (bioNova científica, Madrid, Spain) and the anti-Pgk1p obtained from Alfagene (Molecular probes, Carcavelos, Portugal). The signal was detected with the ECL Amersham kit from GE Healthcare (VWR, Carnaxide, Portugal) and with the Kodak GBX developer and fixer from Sigma-Aldrich (Sintra, Portugal). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat, Marne La Vallée, France), and were normalized against the control sample (DMSO), which was set as 1.

2.8. Co-immunoprecipitation (co-IP) assay

The co-IP assay was performed using the Pierce Classic Magnetic IP and Co-IP Kit from Thermo scientific (Dagma, Carcavelos, Portugal), according to the manufacturer's instructions. Briefly, cells were plated at the final density of 5×10^5 cells/plate and incubated for 24 h. Cells were then treated with 25, 50 and 75 μ M of OXAZ-1 or DMSO only for 16 h. After cell lysis and protein lysate separation, 500 μ g of total protein was incubated with 2 μ L of mouse monoclonal anti-p53 (DO-1) or with

mouse immunoglobulin G (IgG, control) from Santa Cruz Biotechnology (Frilabo, Porto, Portugal), overnight at 4 °C. Immunocomplexes were immunoprecipitated using magnetic beads. Detection of p53, MDM2, MDMX, and GAPDH (loading control) in whole cell lysate (input) and in immunoprecipitated fraction was performed by Western blot analysis as described in section 2.7.

2.9. Analysis of mitochondrial membrane potential ($\Delta\psi_m$)

For analysis of $\Delta\psi_m$, HCT116p53^{+/+} tumor cells were plated in 6-well plates at a final density of 1.5×10^5 cells/well. After 24 h incubation, cells were treated with the GI₅₀ concentration of OXAZ-1 or DMSO only for 8 h. Cells were harvested and incubated with 1 nM DiOC₆(3) from Alfacene (Molecular probes, Carcavelos, Portugal) for 30 min at 37 °C; followed by flow cytometry. Cells treated with 50 μ M carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP; Sigma-Aldrich, Sintra, Portugal), for 15 min at 37 °C, were used as positive control.

2.10. Analysis of reactive oxygen species (ROS) generation

Analysis of intracellular ROS generation was performed by flow cytometry. Briefly, HCT116 tumor cells were plated in 6-well plates at a final density of 1.5×10^5 cells/well and incubated for 24 h. Cells were then treated with the GI₅₀ concentration of OXAZ-1 or DMSO only for 48 h. Cells were harvested and stained with 5 μ M CellROX Green Reagent from Life Technologies (Alfacene, Carcavelos, Portugal) for 30 min at 37 °C.

2.11. Flow cytometric data acquisition and analysis

For the flow cytometric analysis, the Accuri™ C6 flow cytometer from BD Biosciences (Enzifarma, Porto, Portugal) and the CellQuest software from BD Biosciences (Enzifarma, Porto, Portugal) were used. For the identification and quantification of cell cycle phases the ModFit LT software (Verity Software House Inc., Topsham, USA) was used.

2.12. Statistical analysis

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

3. Results

3.1. Identification of OXAZ-1 as potential dual inhibitor of the p53-MDMs interaction, using a yeast-based screening assay

In this work, six (*S*)-tryptophanol-derived oxazolopiperidinone lactams (Fig. 1) were tested for their ability to inhibit the p53 interaction with MDM2 and MDMX, using previously developed yeast-based screening assays [6-8]. These compounds contain an oxazolo ring fused with a piperidinone ring, two scaffolds that are described to act as p53-MDM2 interaction inhibitors [8,13]. Compounds OXAZ-2, OXAZ-3, OXAZ-4, and OXAZ-5 were synthesized by cyclocondensation reaction of (*S*)-tryptophanol and the appropriate δ -oxo-esters [8-10]. The lactams OXAZ-3 and OXAZ-5 were converted to the (*S*)-tryptophanol derivatives OXAZ-6 and OXAZ-1, respectively, with high yields [10].

In the yeast assays used in this work the human p53 retains its function as a transcription factor inducing a marked growth inhibition, which is abolished by human MDM2 and MDMX. As in mammalian cells, inhibitors of the p53-MDMs interaction reduce the impact of MDMs on p53 activity, thus restoring the p53-induced yeast growth inhibition [6-8]. The efficiency of these yeast assays to search for inhibitors of the p53 interaction with MDM2 and MDMX was validated by testing nutlin-3a (a known inhibitor of the p53-MDM2 interaction; [7-8]) and SJ-172550 (a known inhibitor of the p53-MDMX interaction; [6]), respectively. As in mammalian cells [3], also in yeast nutlin-3a did not interfere with the MDMX-inhibitory activity, confirming its activity as MDM2-only inhibitor (Table 1; Fig. 2A). On the other hand, SJ-172550 also did not interfere with the MDM2-inhibitory activity, confirming its activity as MDMX-only inhibitor (Table 1; Fig. 2A). Using this approach, the effect of 0.1 - 50 μ M compounds was evaluated. From the concentration-response curves of the reversion of

MDM2-inhibitory effects obtained for each compound, it was shown that, with the exception of OXAZ-5, all the other tested compounds presented similar effects to nutlin-3a with reversion of the MDM2-inhibitory effect (Table 1). However, only OXAZ-1 was able to revert the MDMX-inhibitory effect, exhibiting a higher potency than SJ-172550 (Table 1). Interestingly, contrary to nutlin-3a and SJ-172550, OXAZ-1 reverted the inhibitory effect of both MDMs (Table 1; Fig. 2A). Additionally, OXAZ-1 did not interfere with the growth of control yeast and yeast expressing p53, MDM2 or MDMX alone (Fig. 2B).

Table 1. EC₅₀ values obtained for the compounds tested in yeast.

Compounds	EC ₅₀ (μM)	
	p53-MDM2	p53-MDMX
Nutlin-3a	1.6 ± 3.6	> 50
SJ-172550	> 50	12.4 ± 4.3
OXAZ-1	1.0 ± 2.4	2.4 ± 3.7
OXAZ-2	2.3 ± 3.7	> 50
OXAZ-3	2.0 ± 3.3	> 50
OXAZ-4	2.1 ± 2.4	> 50
OXAZ-5	> 50	> 50
OXAZ-6	3.0 ± 3.3	> 50

Yeast cells co-expressing p53 and MDM2/MDMX were incubated with 0.1 - 50 μM compounds or DMSO only, for 42 h. The EC₅₀ (concentration that caused 50% of the reversion effect) values were determined from concentration-response curves of the reversion of the MDM2/MDMX-inhibitory effects obtained for 0.1 - 50 μM compounds (see Figure 2A for OXAZ-1 and positive controls). Data are mean ± S.E.M. of six independent experiments.

In a recent work, it was shown that expression of human p53 in yeast increased the expression levels of *ACT1* (described as a yeast p53 target gene; [6]), an effect abolished by MDM2 and MDMX, and re-established by nutlin-3a and SJ-172550, respectively [6]. Here, it was shown that, like nutlin-3a (for MDM2) and SJ-172550 (for MDMX), OXAZ-1 (at 10 μ M for MDM2 and 20 μ M for MDMX) increased the actin protein levels in yeast cells co-expressing p53 and MDM2/MDMX (Fig. 2C). These results reinforced a reversion of the MDMs-inhibitory effect by OXAZ-1.

Double column

Figure 2. Identification of OXAZ-1 as potential dual inhibitor of the p53 interaction with MDM2 and MDMX, using a yeast-based screening assay. (A) Effect of 0.1 – 50 μ M OXAZ-1, nutlin-3a and SJ-172550 on the reversion of p53-induced yeast growth inhibition by MDM2 and MDMX, after 42 h incubation. The growth of yeast cells co-expressing p53 and MDM2/MDMX was evaluated by CFU

counts; results were plotted setting as 100% the growth achieved with yeast cells expressing p53 alone incubated with DMSO only; data are mean $\pm$ S.E.M. of six independent experiments; values significantly different from DMSO only are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Effect of 0.1 – 50 μ M OXAZ-1 on the growth of yeast expressing p53, MDMX, MDM2 or empty vector after 42 h incubation. Yeast cell growth was evaluated by CFU counts; results were plotted setting as 100% the growth achieved with DMSO only; data are mean $\pm$ S.E.M. of five independent experiments; values are not significantly different from DMSO only ($p > 0.05$). (C) Effect of 10 μ M OXAZ-1 on the actin protein levels of yeast cells co-expressing p53 and MDM2/MDMX after 42 h incubation. Immunoblots represent one of three independent experiments; Pgk1p was used as loading control.

3.2. OXAZ-1 exhibits a p53-dependent tumor growth-inhibitory effect mediated by disruption of the p53-MDMs interaction and induction of a mitochondria-mediated apoptotic pathway

The tumor cell growth-inhibitory potential of OXAZ-1 and the contribution of the p53 pathway to its activity were thereafter ascertained using the human colon adenocarcinoma HCT116 cell lines with wt p53 (HCT116 p53^{+/+}) and its p53-null isogenic derivative (HCT116 p53^{-/-}). The GI₅₀ values obtained for OXAZ-1, after 48 h treatment, in p53^{+/+} and p53^{-/-} HCT116 cells, revealed a p53-dependent growth-inhibitory effect by OXAZ-1 (Fig. 3A). In fact, a significant reduction of the potency of OXAZ-1 was observed when the p53 pathway was knocked out in HCT116 p53^{-/-} cells.

In HCT116 p53^{+/+} cells, the growth-inhibitory effect of OXAZ-1, at the GI₅₀ concentration (25 μ M), was associated with G0/G1-phase cell cycle arrest (Fig. 3B) and late apoptosis (Fig. 3C). The activation of a p53-dependent apoptotic pathway by 25 μ M

OXAZ-1 was further supported by the inability of this compound to induce apoptosis (Fig. 3C) and PARP cleavage (Fig. 3D), a product of caspase-3 activation, in p53-null HCT116 cells.

Figure 3. OXAZ-1 induces growth inhibition associated with cell cycle arrest and p53-dependent apoptosis, in human colon adenocarcinoma HCT116 tumor cells. (A) The GI₅₀ concentration of OXAZ-1 was determined in p53^{+/+} and p53^{-/-} HCT116 cells, after 48 h treatment, using the SRB assay. Data are mean ± S.E.M. of four independent experiments; values significantly different from HCT116 p53^{+/+} cells are indicated (***p* < 0.01). (B) 25 μM OXAZ-1 induces G0/G1-phase cell cycle arrest in HCT116 p53^{+/+} cells, after 24 h treatment. Cell cycle phases were analyzed by flow

cytometry using PI staining; data are mean $\pm$ S.E.M. of two independent experiments; values significantly different from DMSO are indicated (** $p < 0.01$, *** $p < 0.001$). (C) 25 μ M OXAZ-1 induces late apoptosis in p53^{+/+}, but not in p53^{-/-}, HCT116 cells, after 24 h treatment. Apoptosis was analyzed by flow cytometry using FITC-Annexin V and PI; data are mean $\pm$ S.E.M. of two independent experiments; values significantly different from DMSO are indicated (*** $p < 0.001$). (D) 25 μ M OXAZ-1 leads to PARP cleavage in p53^{+/+}, but not in p53^{-/-}, HCT116 cells, after 24 h treatments. Immunoblots represent one of three independent experiments; GAPDH was used as loading control.

By western blot analysis, it was also observed that 25 μ M OXAZ-1 led to p53 stabilization and increased the levels of proteins encoded by major p53 transcription targets, such as MDM2, MDMX, p21 (cell cycle regulator), Puma and Bax (apoptotic proteins of the Bcl-2 family), in p53^{+/+}, but not in p53^{-/-}, HCT116 cells (Fig. 4A).

The ability of OXAZ-1 to block the p53 interaction with MDM2 and MDMX was further demonstrated by co-IP, in HCT116 p53^{+/+} cells (Fig. 4B). In fact, at 50 and 75 μ M (corresponding to 2xGI₅₀ and 3xGI₅₀ concentrations, respectively), little or no MDM2 co-immunoprecipitated with p53, and a visible decrease of the amount of MDMX co-immunoprecipitated with p53, were observed (Fig. 4B). Similar results were reported for RO-5963, for which the disruption of the p53 interaction with MDM2 and MDMX was observed at 4xGI₅₀ concentration [3].

1.5 column

Figure 4. OXAZ-1 leads to p53 stabilization and to the up-regulation of p53 target genes by blocking the p53 interaction with MDM2 and MDMX, in HCT116 p53^{+/+} cells. (A) 25 μ M OXAZ-1 increases the protein levels of p53, MDM2, MDMX, p21, Puma and Bax in p53^{+/+}, but not in p53^{-/-}, HCT116 cells after 24 h treatment. Immunoblots represent one of three independent experiments; GAPDH was used as loading control. (B) Cells were treated with 25, 50 and 75 μ M OXAZ-1 or DMSO only for 16 h, followed by immunoprecipitation with p53 or mouse immunoglobulin G (IgG) antibodies, and western blot with MDM2, MDMX and p53 antibodies; whole cell lysate (Input); immunoblots represent one of three independent experiments; GAPDH was used as loading control.

412 Additionally, it was also observed that 25 μM OXAZ-1 led to a marked increase
 413 of ROS production in $p53^{+/+}$, but not in $p53^{-/-}$, HCT116 tumor cells and to $\Delta\psi_m$
 414 dissipation in HCT116 $p53^{+/+}$ cells (Fig. 5A). Moreover, besides the increase of Bax
 415 production (Fig. 4A), 25 μM OXAZ-1 triggered the translocation of this mitochondrial
 416 protein from the cytosol to mitochondria (Fig. 5B), and the release of cytochrome *c* (cyt
 417 *c*) from mitochondria to cytosol (Fig. 5C). Altogether, these results showed that OXAZ-
 418 1 was a potent inducer of a p53-dependent mitochondria-mediated apoptotic pathway in
 419 human tumor cells.

Figure 5. OXAZ-1 induces a p53-dependent mitochondria-mediated apoptotic pathway. (A) 25 μ M OXAZ-1 induces ROS production in p53^{+/+}, but not in p53^{-/-}, HCT116 cells after 48 h treatment, and $\Delta\psi_m$ dissipation in HCT116 p53^{+/+} cells after 8 h treatment. For assessment of ROS production and $\Delta\psi_m$, cells were stained with CellROX and DIOC₆(3), respectively, and analyzed by flow cytometry; histograms represent one of two independent experiments; M2 cursor indicates the subpopulation analyzed; represented values correspond to the increase in the percentage of CellROX-positive cells and of cells with $\Delta\psi_m$ dissipation obtained after treatment with OXAZ-1 or DMSO only, and are mean $\pm$ S.E.M. of two independent experiments; in $\Delta\psi_m$ analysis, FCCP was used as positive control. (B) 25 μ M OXAZ-1 induces Bax translocation from cytosol to mitochondria after 24 h treatment. GAPDH and COX IV were used as loading control of cytosolic and mitochondrial fractions, respectively. (C) 25 μ M OXAZ-1 triggers mitochondrial cyt *c* release into the cytosol after 24 h treatment. GAPDH was used as loading control. In B and C, immunoblots represent one of three independent experiments.

3.3. MDMX-overexpression tumor cells are sensitive to OXAZ-1

Tumor cells overexpressing MDMX have been described as fairly insensitive to MDM2-only inhibitors (e.g., nutlin-3a) due to their inability to inhibit the p53-MDMX interaction [3,14-15]. Based on this, the effect of OXAZ-1 was tested in human breast cancer MCF-7 cells, a well-known MDMX-overexpressing tumor cell line [3]. As expected, a similar growth-inhibitory effect to that obtained in HCT116 p53^{+/+} cells was achieved in MCF-7 cell lines by OXAZ-1 (GI₅₀ value of 23.5 ± 2.5 μ M). Additionally, as in HCT116 p53^{+/+} cells, also in MCF-7 tumor cell lines, the OXAZ-1-induced growth

inhibition was associated with G0/G1-phase cell cycle arrest (Fig. 6A) and late apoptosis (Fig. 6B) with PARP cleavage (Fig. 6C). Moreover, also in MCF-7 tumor cell lines, 23.5 μ M OXAZ-1 led to the stabilization of p53 protein levels and to the up-regulation of p53 transcription targets involved in cell cycle (p21) and apoptosis (Bax) (Fig. 6D). Altogether, these results showed that also in MDMX-overexpression tumor cells OXAZ-1 was able to activate the p53 pathway mostly inhibited by MDMX.

451

452

Double column

Figure 6. OXAZ-1 induces growth inhibition associated with cell cycle arrest and apoptosis in MDMX-overexpressing human breast adenocarcinoma MCF-7 tumor cells through activation of the p53 pathway. (A) 47 μ M OXAZ-1 induces G0/G1-phase cell cycle arrest in MCF-7 cells, after 24 h treatment. Cell cycle phases were analyzed by flow cytometry using PI staining; data are mean $\pm$ S.E.M. of two independent experiments; values significantly different from DMSO are indicated (** p < 0.01). (B) 47 μ M OXAZ-1 induces late apoptosis in MCF-7 cells, after 24 h

treatment. Apoptosis was analyzed by flow cytometry using FITC-Annexin V and PI; data are mean $\pm$ S.E.M. of two independent experiments; values significantly different from DMSO are indicated (* $p < 0.05$). (C, D) 23.5 μ M OXAZ-1 leads to PARP cleavage (C), and increases the protein levels of p53, p21 and Bax (D) in MCF-7 tumor cells, after 24 h treatments. Immunoblots represent one of three independent experiments; GAPDH was used as loading control.

3.4. OXAZ-1 sensitizes tumor cells to conventional chemotherapeutic drugs in a p53-dependent manner

It was also investigated if OXAZ-1 increased the sensitivity of tumor cells to the effects of conventional chemotherapeutic drugs, such as doxorubicin and etoposide. For that, the tumor growth-inhibitory effect of a very low concentration of OXAZ-1 (GI_{10} of 9 μ M; for which no significant effects on tumor cell growth were observed), combined with increasing concentrations of doxorubicin and etoposide, was investigated in HCT116 cells. The results obtained revealed pronounced synergistic effects between OXAZ-1 and the tested conventional chemotherapeutic drugs in p53^{+/+}, but not in p53^{-/-}, HCT116 cells (Fig. 7A-C). In fact, in HCT116 p53^{+/+} cells, the Q values obtained, at the three tested concentrations of doxorubicin and etoposide, were higher than 1.15 ($1.43 < Q < 2.22$ for doxorubicin; $1.64 < Q < 2.22$ for etoposide) (Fig. 7A). Interestingly, the best result obtained corresponded to the lowest concentrations tested of doxorubicin and etoposide. Accordingly, synergistic effects between OXAZ-1 and the lowest concentrations tested of doxorubicin (9.38 nM) and etoposide (0.38 μ M) were obtained in the analysis of total apoptosis by Annexin V in HCT116 p53^{+/+} cells (Fig. 7B). Contrary, OXAZ-1 did not significantly interfere with the tumor growth-inhibitory effect of doxorubicin and etoposide for the three tested concentrations of these

485 chemotherapeutic drugs in p53-null HCT116 cells (Fig. 7C). Altogether, the results
 486 obtained showed a p53-dependent synergistic effect of OXAZ-1 with conventional
 487 chemotherapeutic drugs.

488

489

Double column

490 **Figure 7. OXAZ-1 sensitizes HCT116 p53^{+/+} tumor cells to the effects of**
 491 **doxorubicin and etoposide.** (A) HCT116 p53^{+/+} tumor cells were treated with
 492 increasing concentrations of doxorubicin (9.38 to 37.5 nM) or etoposide (0.38 to 3.00
 493 μ M) and 9 μ M (GI₁₀ concentration) of OXAZ-1 or DMSO only (control). Effect of

494 compounds on the tumor cell growth was analyzed after 48 h incubation, using the SRB
495 assay; the percentage of cell growth achieved with OXAZ-1 only was 90.6%; data are
496 mean $\pm$ S.E.M. of four independent experiments. **(B)** Effect of combined treatment on
497 apoptosis was determined after 48 h treatment with a fixed concentration of doxorubicin
498 (9.38 nM) or etoposide (0.38 μ M) and the GI₁₀ concentration of OXAZ-1 or DMSO
499 only (control). The percentage of apoptotic cells achieved with OXAZ-1 only was 1.5%;
500 apoptosis was analyzed by flow cytometry using FITC-Annexin V and PI; data are
501 mean $\pm$ S.E.M. of three to four independent experiments. **(C)** HCT116 p53^{-/-} tumor cells
502 were treated with increasing concentrations of doxorubicin (18.75 to 150 nM) or
503 etoposide (0.75 to 6.00 μ M) and 9 μ M OXAZ-1 or DMSO only (control). Effect of
504 compounds on tumor cell growth was analyzed after 48 h incubation, using the SRB
505 assay; the percentage of cell growth achieved with OXAZ-1 only was approximately
506 100%; data are mean $\pm$ S.E.M. of four independent experiments. In **A** and **B**, the Q
507 values are indicated for combined treatments of OXAZ-1/doxorubicin and OXAZ-
508 1/etoposide; $Q < 0.85$, $Q > 1.15$ and $0.85 < Q < 1.15$ indicate antagonism, synergy and
509 additive effects, respectively; dotted line represents $Q = 1.15$.

4. Discussion

In this work, a yeast-based screening assay was used to search for inhibitors of the p53 interaction with MDM2 and MDMX. From the analysis of a small library of enantiopure tryptophanol-derived oxazolopiperidone lactams, the (*S*)-tryptophanol derivative OXAZ-1 was identified as potential dual inhibitor of the p53-MDMs interaction. The co-IP assay in human tumor cell lines supported the results from yeast, showing that OXAZ-1 was able to block the MDM2 and MDMX interaction with p53. Beyond that, it was shown that the *in vitro* tumor growth-inhibitory effect of OXAZ-1 was highly dependent on p53 and exhibited typical hallmarks of an inhibitor of the p53 interaction with MDM proteins [2]. Particularly, it led to p53 stabilization, what is consistent with an inhibition of the MDM2-mediated p53 degradation by OXAZ-1, and up-regulated p53 transcription targets, such as MDM2, MDMX, p21, Bax and Puma. Additionally, like RO-5963 [3], OXAZ-1 efficiently reduced the proliferation of wt p53-carrying tumor cells with high levels of MDMX (MCF-7 cells). In fact, also in MDMX-overexpression tumor cells, OXAZ-1 induced cell cycle arrest and apoptosis through activation of the p53 transcriptional activity. OXAZ-1 therefore overcame the commonly reported [3, 14-15] resistance of MDMX-overexpression tumors to MDM2-only inhibitors.

Cancer cell mitochondria are structurally and functionally different from their normal counterparts. Additionally, tumor cells are more susceptible to mitochondrial perturbations than normal cells. Based on this, mitochondrially-targeted agents have emerged as a promising approach to selectively eradicate chemotherapy-refractory cancer cells [16]. In fact, the activation of cell death programmes by pharmacological agents that induce or facilitate mitochondrial membrane permeabilization (MMP) has emerged as an attractive strategy for cancer treatment [16]. MMP can be triggered by

agents that increase cytosolic calcium or stimulate ROS generation. Moreover, MMP can be favored by pro-apoptotic proteins of the Bcl-2 family, such as Bax. In fact, during apoptosis, Bax is translocated from the cytosol to mitochondria where it triggers MMP [16,17]. MMP results in the immediate $\Delta\psi_m$ dissipation, with the consequent release of pro-apoptotic factors into the cytosol, such as cyt *c* [16]. The results obtained in the present work showed that OXAZ-1 also targets the mitochondria of tumor cells. Actually, OXAZ-1 potently triggered a mitochondrion-centered p53-dependent apoptotic cell death characterized by ROS generation, Bax translocation to mitochondria, $\Delta\psi_m$ dissipation, and mitochondrial cyt *c* release.

The pharmacological activation of the p53 pathway can be exploited to work in combination with other therapeutic agents either to protect from side effects of chemotherapy [18], or to promote cell death via p53-dependent and -independent mechanisms [19]. Concerning to synergistic enhancement of cell death, it is still not completely understood how drug combinations with p53 activators can improve therapeutic efficacy in cancer treatment [19]. In spite of this, examples were already reported showing the efficacy of such combinations in anticancer treatment. This is the case of nutlin-3a, which has shown excellent results when combined with non-targeted genotoxic agents [2,19]. In this work, very low concentrations of OXAZ-1 exhibited pronounced p53-dependent synergistic effects with etoposide and doxorubicin. The potential antitumor activity of OXAZ-1 is therefore strengthened in this work by showing that this compound sensitizes tumor cells to the effects of chemotherapeutic drugs. Thus, the prospect of combining conventional chemotherapeutic agents with OXAZ-1 in cancer therapy represents a promising strategy to minimize the emergence of resistance and to achieve maximal therapeutic responses with minimal side effects.

5. Conclusion

Although further work is still required to completely clarify the molecular targets of (*S*)-tryptophanol derivative OXAZ-1, particularly its interaction with MDM2 and MDMX, relevant insights about its mode of action are already provided in this study (Fig. 8). This work opens the way to a new class of selective activators of the p53 pathway, based on a tryptophanol-derived oxazolopiperidone lactam scaffold, with promising antitumor properties either isolated or in combined therapies. Additionally, the identification of (*S*)-tryptophanol derivative OXAZ-1 may be the starting basis toward the development of promising dual inhibitors of the p53 interaction with MDM proteins.

Figure 8. Proposed molecular mechanism of action of (S)-tryptophanol derivative OXAZ-1 in human tumor cells. As dual inhibitor of the p53 interaction with MDM2 and MDMX, OXAZ-1 leads to p53 stabilization and to the activation of p53 transcriptional activity, increasing the levels of the p53 target proteins MDMX, MDM2, p21, Bax and Puma. The increase of p21 levels leads to a G0/G1 cell cycle arrest. The increase of Puma and Bax leads to the activation of a mitochondrial apoptotic pathway, involving Bax translocation to mitochondria, ROS generation, $\Delta\psi_m$ dissipation, and cytochrome *c* release into cytosolic fraction. The release of cytochrome *c* from mitochondria triggers the activation of a caspase pathway with PARP cleavage; *p53 target genes.

Acknowledgments

This work is funded by FEDER funds through the Operational Programme for Competitiveness Factors - COMPETE and by National Funds through FCT - Foundation for Science and Technology under the PEST-C/EQB/LA0006/2013 (REQUIMTE) and Pest-OE/SAU/UI4013/2014 (iMed.U LISboa), and through the research projects PTDC/QUI-QUI/111664/2009 and PTDC/SAU-FAR/110848/2009. It was also supported by the FCT fellowships of J. Soares (SFRH/BD/78971/2011) and M. Leão (SFRH/BD/64184/2009). Thanks are also due to the MINECO, Spain (project CTQ2012-35250).

References

- [1] Girardini JE, Marotta C, Del Sal G. Disarming mutant p53 oncogenic function. *Pharmacol Res.* 2014;79:75-87.
- [2] Wade M, Li YC, Wahl GM. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer* 2013;13:83-96.
- [3] Graves B, Thompson T, Xia M, Janson C, Lukacs C, Deo D, Di Lello P, Fry D, Garvie C, Huang KS, Gao L, Tovar C, Lovey A, Wanner J, Vassilev LT. Activation of the p53 pathway by small-molecule-induced MDM2 and MDMX dimerization. *PNAS* 2012;109:11788-93.
- [4] Lee JH, Zhang Q, Jo S, Chai SC, Oh M, Im W, Lu H, Lim HS. Novel pyrrolopyrimidine-based alpha-helix mimetics: cell-permeable inhibitors of protein-protein interactions. *J Am Chem Soc* 2011;133:676-9.
- [5] Qin L, Yang F, Zhou C, Chen Y, Zhang H, Su Z. Efficient Reactivation of p53 in Cancer Cells by a Dual MdmX/Mdm2 Inhibitor. *J Am Chem Soc* 2014;136:18023-33.
- [6] Leão M, Gomes S, Soares J, Bessa C, Maciel C, Ciribilli Y, Pereira C, Inga A, Saraiva, L. Novel simplified yeast-based assays of regulators of p53-MDMX interaction and p53 transcriptional activity. *FEBS J* 2013;280:6498-507.
- [7] Leão M, Pereira C, Bisio A, Ciribilli Y, Paiva A, Machado N, Palmeira A, Fernandes MX, Sousa E, Pinto M, Inga A, Saraiva L. Discovery of a new small-molecule inhibitor of p53-MDM2 interaction using a yeast-based approach. *Biochem Pharmacol* 2013;85:1234-45.
- [8] Soares J, Pereira NAL, Monteiro A, Leão M, Bessa C, dos Santos JVA, Raimundo L, Queiroz G, Bisio A, Inga A, Pereira C, Santos MMM, Saraiva L. Oxazoloisoindolinones with in vitro antitumor activity selectively activate a p53-

pathway through potential inhibition of the p53-MDM2 interaction. *Eur J Pharm Sci* 2015;13:138-47.

[9] Amat M, Santos MMM, Bassas O, Llor N, Escolano C, Gómez-Esqué A, Molins E, Allin SM, McKee V, Bosch J. Straightforward methodology for the enantioselective synthesis of benzo[*a*]- and indolo[2,3-*a*]quinolizidines. *J Org Chem* 2007;72:5193-201.

[10] Amat M, Santos MMM, Gómez AM, Jokic D, Molins E, Bosch J. Enantioselective spirocyclizations from tryptophanol-derived oxazolopiperidone lactams. *Org Lett* 2007;9:2907-10.

[11] Pereira NAL, Sureda FX, Espuglas R, Pérez M, Amat M, Santos MMM. Tryptophanol-derived oxazolopiperidone lactams: identification of a hit compound as NMDA receptor antagonist. *Bioorg Med Chem Lett* 2014;24:3333-36.

[12] Jin ZJ. Addition in drug combination (author's transl). *Acta Pharmacol Sin* 1980;1:70-6.

[13] Sun D, Li Z, Rew Y, Gribble M, Bartberger MD, Beck HP, Canon J, Chen A, Chen X, Chow D, Deignan J, Duquette J, Eksterowicz J, Fisher B, Fox BM, Fu J, Gonzalez AZ, Gonzalez-Lopez De Turiso F, Houze JB, Huang X, Jiang M, Jin L, Kayser F, Liu JJ, Lo MC, Long AM, Lucas B, McGee LR, McIntosh J, Mihalic J, Oliner JD, Osgood T, Peterson ML, Roveto P, Saiki AY, Shaffer P, Toteva M, Wang Y, Wang YC, Wortman S, Yakowec P, Yan X, Ye Q, Yu D, Yu M, Zhao X, Zhou J, Zhu J, Olson SH, Medina JC. Discovery of AMG 232, a Potent, Selective, and Orally Bioavailable MDM2-p53 Inhibitor in Clinical Development. *J Med Chem* 2014;57:1454-72.

[14] Patton JT, Mayo LD, Singhi AD, Gudkov AV, Stark GR, Jackson MW. Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. *Cancer Res* 2006;66:3169-76.

637 [15] Wade M, Wong ET, Tang M, Stommel JM, Wahl GM. Hdmx modulates the
638 outcome of p53 activation in human tumor cells. *J Biol Chem* 2006;281:33036-44.

639 [16] Fulda S, Gorman AG, Hori O, Samali A. Cellular Stress Responses: Cell Survival
640 and Cell Death. *Inter J Cell Biol* 2010;2010:1-23.

641 [17] Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M,
642 Green DR. Direct activation of Bax by p53 mediates mitochondrial membrane
643 permeabilization and apoptosis. *Science* 2004;303:1010-14.

644 [18] Wang X, Zhu S, Qian L, Gao J, Wu M, Gao J, Zhang Y, Chan GL, Yu Y, Han W.
645 IL-1Ra selectively protects intestinal crypt epithelial cells, but not tumor cells, from
646 chemotoxicity via p53-mediated upregulation of p21(WAF1) and p27(KIP1.).
647 *Pharmacol Res.* 2014;82:21-33.

648 [19] Hoe KK, Verma CS, Lane DP. Drugging the p53 pathway: understanding the route
649 to clinical efficacy. *Nat Rev Drug Discov* 2014;13:217-36.

TRYPTOPHANOL-DERIVED OXAZOLOPIPERIDONE LACTAM: OXAZ-1

DUAL P53-MDM2/MDMX INHIBITOR

MITOCHONDRIA-MEDIATED APOPTOSIS

SYNERGISTIC EFFECT