

OXAZ-1: a new small molecule with *in vitro* antitumor activity through selective activation of a mitochondria-mediated p53 pathway and potential MDM2/MDMX inhibition

ABSTRACT

Due to the critical and non-redundant role of MDM2 and MDMX in inactivating the wild-type (wt) p53 tumour suppressor protein, the simultaneous inhibition of their interactions with p53, for a full p53 reactivation, represents a promising anticancer strategy. Here, we report the identification of a potential dual inhibitor of the p53 interaction with MDM2 and MDMX, the *N*-tosylindole OXAZ-1, from the screening of a small library of tryptophanol-derived oxazolopiperidone lactams, using a yeast assay. Using human colon adenocarcinoma HCT116 cells with wt p53 (HCT116 p53^{+/+}) and its p53-null isogenic derivative (HCT116 p53^{-/-}), it was shown that OXAZ-1 had a p53-dependent *in vitro* antitumor activity. Additionally, OXAZ-1 led to p53 stabilization, increased the protein levels of the p53 transcription targets MDM2, MDMX, p21, Puma and Bax, and induced PARP cleavage, in p53^{+/+}, but not in p53^{-/-}, HCT116 cells. In HCT116 p53^{+/+} cells, OXAZ-1 potently triggered a mitochondria-mediated apoptotic cell death characterized by reactive oxygen species generation, Bax translocation to mitochondria, mitochondrial membrane potential dissipation, and mitochondrial cytochrome *c* release. A similar antitumor activity of OXAZ-1 was observed against MDMX-overexpressing breast adenocarcinoma MCF-7 tumour cells. OXAZ-1 was also highly effective in combination with conventional chemotherapies increasing the antitumor outcome at very low concentrations. Finally, computational docking studies supported that OXAZ-1 binds to MDM2 and MDMX.

Altogether, this study reports the identification of a selective activator of the p53 pathway and may open the way to the development of a new class of dual MDM2/MDMX inhibitors with promising applications in anticancer therapy.

1. Introduction

The tumour suppressor p53 is a major transcription factor activated in response to cellular stresses to induce cell cycle arrest, senescence, and apoptosis. *TP53* is the most frequently mutated gene in human cancers (Hoe et al., 2014). Additionally, in tumours retaining a wild-type (wt) p53 status, the activity of this protein is suppressed due to the overexpression of two structurally related p53-negative regulators, murine double minute (MDM)2 and MDMX. The p53-binding domains at the N-termini of MDM2 and MDMX interacts with the transactivation domain of p53 repressing its transcriptional activity. At the C-termini of both proteins is located the RING-finger domain, which serves as specific E3-ubiquitin ligase in MDM2, but not in MDMX. The E3-ubiquitin ligase MDM2 binds to the N-terminus of p53, disabling its transcriptional activity and targeting it for ubiquitylation and subsequent proteasome-mediated degradation. On the other hand, MDMX has no ubiquitylation activity, but it can enhance the ligase activity of MDM2 toward p53 by heterodimerization with MDM2 (Li and Lozano, 2013; Wade et al., 2013; Hoe et al., 2014). Accumulating data have shown that MDM2 and MDMX function in a non-overlapping manner, creating together a negative feedback loop, which precisely controls the levels and activity of p53. Based on this, the disruption of the p53-MDMs interaction to reactivate p53 represents a valuable therapeutic strategy for tumour treatment, either individually or combined with conventional chemotherapeutic agents (Li and Lozano, 2013; Mir et al., 2013; Wade et al., 2013; Hoe et al., 2014).

To date, most of the pharmacological efforts have been focused on MDM2. However, despite the huge number of small molecule inhibitors of the p53-MDM2 interaction that have been described so far, just few have reached clinical trials, such as RG7112 (a cis-imidazoline compound from the nutlin family) (Ray-Coquard et al.,

2012; Hoe et al., 2014), and MI-773 (a spiro-oxindole compound) (Hoe et al., 2014). Additionally, due to differences in their p53-binding pockets, small molecule MDM2 inhibitors have shown very low binding affinity to MDMX (Li and Lozano, 2013). Accordingly, just recently, the first small molecule inhibitor of the p53-MDMX interaction, called SJ-172550 (Reed et al., 2010), and dual inhibitor of MDM2 and MDMX, called RO-5963 (Graves et al., 2012), were identified.

Several strategies have been used to discover small molecule inhibitors of the p53-MDMs interaction, such as the structure-based design and the high-throughput screening of libraries of compounds (Hoe et al., 2014). Recently, our group developed a new cell-based approach, based on yeast cells co-expressing human p53 with human MDM2 or MDMX, for the directed screening of inhibitors of the p53 interaction with MDM2 (Leão et al., 2013a) and MDMX (Leão et al., 2013b). The use of this yeast screening approach led us to the recent identification of a new small molecule inhibitor of the p53-MDM2 interaction with a xanthone scaffold (Leão et al., 2013a). With that study, a proof of concept was provided for the efficacy of this cell system for the screening of inhibitors of the p53-MDMs interaction.

In this work, from the screening of a small library of tryptophanol-derived oxazolopiperidone lactams using the yeast approach, it was identified the small molecule *N*-tosylindole OXAZ-1 (Figure 1), which selectively activates the p53 pathway through potential inhibition of MDM2 and MDMX. Moreover, OXAZ-1 exhibits a p53-dependent *in vitro* antitumor activity against tumours retaining wt p53 and distinct levels of MDM2 and MDMX. The promising antitumor activity of OXAZ-1 is reinforced by its ability to trigger a mitochondria-mediated apoptotic cell death and to sensitize tumour cells to conventional chemotherapeutic drugs.

2. Methods

2.1 Compounds

OXAZ-2, OXAZ-3, OXAZ-4, and OXAZ-5, whose molecular structures are shown in Fig. 1 were synthesized according to described procedures (Amat et al, Journal Organic Chemistry 2007 and Organic Letters 2007, Santos et al, Bioorganic Medicinal Chemistry Letters 2014). 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).

2.1.1. General procedure for the synthesis of OXAZ-1 and OXAZ-6

A solution of the adequate indole-oxazolopiperidone lactam in CH_2Cl_2 was cooled to 0°C . *p*-Toluenesulphonyl Chloride (1.2eq.) and Tetrabutylammonium Chloride (cat., 10%mmol) were added and the mixture was stirred for 10min. Then, an aqueous solution of NaOH (30% m/v, 0.5v CH_2Cl_2) was added and the reaction was allowed to stand at room temperature for 24h. After this period, the reaction was diluted with CH_2Cl_2 and the phases were separated. The organic phase was washed with HCl (1M) and dried over Na_2SO_4 . After solvent evaporation the crude compound was purified by flash chromatography with the adequate eluent system (Amat et al, OL 2007).

2.2. Plasmids

The yeast expression vectors were used: pGADT7-(*LEU2*) encoding human MDM2 (kindly provided by Dr Xue-Min Zhang; National Center of Biomedical Analysis, China) or MDMX (kindly provided by Dr Martin Scheffner; University of

Konstanz, Germany) under *ADHI* constitutive promoter; pLS89-(*TRP1*) encoding human wt p53 under *GALI-10* inducible promoter (kindly provided by Dr Richard Iggo; Swiss Institute for Experimental Cancer Research, Switzerland).

2.3. Yeast target-directed screening assay

Saccharomyces cerevisiae (strain CG379) expressing human wt p53 alone and combined with human MDM2 or MDMX were obtained in previous works (Leão et al., 2013a,b). For expression of human proteins, cells (routinely grown in minimal selective medium) 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 pLS89 and pGADT7 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 tumour cell lines and growth conditions

The human colon adenocarcinoma HCT116 cell line harbouring a wt p53 form (HCT116 p53^{+/+}), and its isogenic derivative in which p53 has been knocked out (HCT116 p53^{-/-}), and the human breast adenocarcinoma MCF-7 tumour cell line were used. 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 analyses of the effect of compounds on the *in vitro* growth of human tumour 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 compound (from 1.85 to 150 μ M). The effect of the compounds was analysed 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 compounds (DMSO) corresponding to the maximum concentration used in these assays (0.25%) was included as control. The concentration of compound that causes a 50% reduction in the net protein increase in cells during treatment (GI₅₀, growth inhibition of 50%) was determined for all tested compounds.

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

HCT116 p53^{+/+} and MCF-7 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 and with twice the GI₅₀ concentration (2xGI₅₀) of OXAZ-1 or DMSO only for 24 h. For cell cycle analysis, cells were thereafter fixed in ice-cold 70% ethanol and incubated at 37 °C with RNase A from Sigma-Aldrich (Sintra, Portugal) at a final concentration of 20 μ g/mL for 15 min, and further incubated with 50 μ g/mL propidium iodide (PI) from

Fluka (Sigma-Aldrich, Sintra, Portugal) for 30 min, followed by flow cytometric analysis. For apoptosis analysis, cells were analyzed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit I from BD Biosciences (Enzifarma, Porto, Portugal), according to the manufacturer's instructions.

2.7. Western blot analysis

To prepare whole protein extracts from yeast, these cells were lysed with Cellytic™ Y Cell Lysis Reagent containing EDTA-free protease inhibitor cocktail from Sigma-Aldrich (Sintra, Portugal). To prepare the whole protein extracts from human tumour cell lines, cells were lysed with RIPA buffer containing EDTA-free protease inhibitor cocktail from Sigma-Aldrich (Sintra, Portugal). For mitochondrial and cytosolic fractions of human tumour cell lines, the Mitochondrial Fractionation Kit from Active Motif (Frilabo, Porto, Portugal) was used according to the manufacturer's instructions.

Whole protein extracts were quantified using the Coomassie staining Bradford from Sigma-Aldrich (Sintra, Portugal). Proteins (40 µg) were electrophoresed using a 10% SDS-PAGE and transferred to a Whatman 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) followed by an anti-mouse horseradish-peroxidase (HRP)-conjugated secondary antibody. For p21, MDMX and actin detection, membranes were probed with a rabbit polyclonal anti-p21 (C-19), anti-MDMX (A300), and anti-actin (C11), respectively, followed by an 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 analyses of mitochondrial and cytosolic fractions, membranes were reprobed with the loading controls mouse monoclonal anti-GAPDH (6C5) or anti-Cox4 (F-8), used to exclude putative contamination of cytosolic and mitochondrial fractions, respectively. All antibodies were purchased from Santa Cruz Biotechnology (Frilabo, Porto, Portugal), except the anti-MDMX from Bethyl Laboratories (bioNova científica, Madrid, Spain) and the anti-Pgk1p from Alfacene (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).

2.8. Analysis of mitochondrial transmembrane potential ($\Delta\psi_m$)

For analysis of $\Delta\psi_m$, HCT116 p53^{+/+} tumour 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; cells treated with 50 μ M carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP; Sigma-Aldrich, Sintra, Portugal), for 15 min at 37 °C, were used as positive control of depolarised cells.

2.9. Analysis of reactive oxygen species (ROS) production

Analysis of intracellular ROS generation was performed by flow cytometry. Briefly, HCT116 p53^{+/+} tumour 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 (Alfagene, Carcavelos, Portugal) for 30 min at 37 °C.

2.10. 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.

2.11. Computational chemistry

The OXAZ-1 chemical structure was built with MOE and energetically minimized using the MMFF94x force field with a RMS gradient of 0.1. The crystallographic structure of MDM2 protein bound to the MI-63 analog (PDB code: 3LBL) was imported in MOE and the co-crystallized waters were removed. Only the C chain was kept and the residues were protonated to match their state at physiological pH using the protonate3D module of MOE. The MDM2 residues around 4.5 Å of MI-6 were selected to define the binding pocket.

For MDMX, a similar procedure was used with the 3LBJ crystallographic structure by removing extra co-crystallized molecules but retaining the WK298 inhibitor located in the p53 experimental docking cleft.

All docking experiments were performed using a triangle matcher placement with a London dG scoring and a MMFF94x force field refinement followed by a GBVI/WSA dG rescoring (defaults in MOE 2013.08). This protocol was tested by re-docking the co-

crystallized inhibitors (MI-63 analog in MDM2 and WK298 in MDMX). The top ranked poses were very similar to the experimental ones and correctly reproduced the main interaction features (as expected, the largest variation was on the terminal carbon chain of WK298).

The results were ranked through their binding affinities and the molecular interactions were interpreted based on the best scored conformation per structure.

2.12. Statistical analysis

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

Results

Identification of OXAZ-1 as potential dual inhibitor of the p53 interaction with MDM2 and MDMX in yeast

Six oxazolopiperidinone lactams (Figure 1) were evaluated as dual inhibitors of the p53 interaction with MDM2 and MDMX using yeast target-directed screening assays. These compounds contain in the same structure two privileged scaffolds in MDM2 inhibitors design: the piperidinone and indole skeletons.

Compounds OXAZ-2, OXAZ-3, OXAZ-4, and OXAZ-5 were synthesised by cyclocondensation reaction of *S*-tryptophanol and the appropriate δ -oxo-esters (Amat et al, JOC 2007 and Santos et al, BMCL 2014). The lactams OXAZ-3, and OXAZ-5 were converted to the *N*-tosyl indole derivatives OXAZ-6, and OXAZ-1, respectively, in good yields.

The effect of the synthesized compounds as inhibitors of the p53-MDM2/X interaction was thereafter tested using previously developed yeast target-directed screening assays (Leão et al., 2013a,b). These yeast assays are based on the fact that human p53 is also a transcription factor in yeast inducing a marked growth inhibition, which is abolished by human MDM2 and MDMX (Figure 2A). 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 (Figure 2A). 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; Vassilev et al., 2004) and SJ-172550 (a known inhibitor of the p53-MDMX interaction; Reed et al., 2010), respectively. As in mammalian cells (Graves et al., 2012), also in yeast nutlin-3a did not interfere with the MDMX inhibitory activity,

confirming its activity as MDM2-only inhibitor (Table 1; Figure 2B). On the other hand, SJ-172550 did not interfere with the MDM2 inhibitory activity, only reverting the MDMX inhibitory effect (Table 1; Figure 2B).

Using this yeast approach, the effect of 0.1 - 50 μ M compounds was evaluated. From the concentration-response curves of the reversion of the MDM2 and MDMX 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, reverting the MDM2 inhibitory effect, and therefore behaved as potential inhibitors of the p53-MDM2 interaction (Table 1). However, only OXAZ-1 was able to also revert the MDMX inhibitory effect, exhibiting a higher potency than SJ-172550 (Table 1). In fact, contrary to nutlin-3a and SJ-172550, OXAZ-1 reverted the inhibitory effects of both MDMs (Table 1; Figure 2B), without interfering with the growth of control yeast and yeast expressing p53 alone (Figure 2C).

In a recent work, it was shown that human p53 also increased the expression levels of *ACT1*, a p53 target gene in yeast (Leão et al., 2013b), an effect abolished by MDM2 and MDMX, and re-established by nutlin-3a and SJ-172550, respectively (Leão et al., 2013b). Here, it was shown that the treatment of yeast cells co-expressing p53 with MDM2 or MDMX with OXAZ-1 (10 μ M for MDM2 and 20 μ M for MDMX) increased the actin protein levels, when compared to yeast treated with DMSO only (Figure 2D).

Together, the results obtained in yeast indicated that *N*-tosylindole OXAZ-1 was a potential dual inhibitor of the p53 interaction with MDM2 and MDMX. With the absence of a direct effect of OXAZ-1 on wt p53 activity, the results obtained may indicate that as inhibitor of p53-MDM2/X interaction, OXAZ-1 may act on MDM2 and MDMX.

OXAZ-1 selectively activates the p53 pathway in human tumour cells expressing wt p53

The tumour 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, of 25 µM in HCT116 p53^{+/+} cells and of 66.3 µM in HCT116 p53^{-/-} cells (Figure 3A) revealed a p53-dependent growth inhibitory effect by OXAZ-1. 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 and twice the GI₅₀ (2xGI₅₀) concentration, was associated to G0/G1-phase cell cycle arrest (Figure 3B) and to late apoptosis (Figure 3C). The activation of a p53-dependent apoptotic pathway by OXAZ-1 was also supported by the occurrence of an increase of cleaved PARP, a product of caspase-3 activation, in p53^{+/+}, but not in p53^{-/-}, HCT116 cells (Figure 3D).

To further ascertain whether OXAZ-1 had effects on tumour cells consistent with an inhibition of the p53 interaction with MDM2 and MDMX, the protein levels of p53 and of p53 target genes were checked by Western blot analysis in p53^{+/+} and p53^{-/-} HCT116 cells. The obtained results showed an increase of the p53 baseline levels in HCT116 p53^{+/+} cells upon 25 µM OXAZ-1 treatment (Figure 3E). Moreover, 25 µM OXAZ-1 increased the protein levels of major p53 transcription targets in p53^{+/+}, but not in p53^{-/-}, HCT116 cells, namely of MDM2, MDMX (recently recognised as a p53 target gene; Li et al., 2010; Phillips et al., 2010), the cell cycle regulator p21, and the apoptotic

proteins of the Bcl-2 family, Puma and Bax (Figure 3E). Together, these results showed that OXAZ-1 selectively activated the p53 pathway. Additionally, in tumour cells with a decreased wt p53 activity by MDM2 and MDMX, OXAZ-1 reduced the MDM2-mediated p53 degradation and the MDM2- and MDMX-inhibitory effect on p53 transcriptional activity, leading to the subsequent up-regulation of p53 target genes, which supports a potential inhibition of MDM2 and MDMX by OXAZ-1.

In addition, in HCT116 p53^{+/+} tumour cells, it was observed that 25 μ M OXAZ-1 led to a marked increase of ROS production and $\Delta\psi_m$ dissipation (Figure 4A). Moreover, besides the increase of Bax production, 25 μ M OXAZ-1 triggered the translocation of this mitochondrial protein of the Bcl-2 family from the cytosol to mitochondria (Figure 4B), a typical event of the mitochondrial apoptotic pathway. Finally, an increase of the cytochrome *c* (cyt *c*) levels was observed in the cytosolic fraction of tumour cells after 24 h treatment with 25 μ M OXAZ-1, indicating its release into the cytosol from mitochondria (Figure 4C). Together, these results showed that OXAZ-1 was a potent inducer of a mitochondrial-dependent apoptotic cell death in human tumour cells.

MDMX-overexpression tumour cells are highly sensitive to OXAZ-1

Tumour cells overexpressing MDMX have been described as fairly insensitive to MDM2-only inhibitors (e.g. nutlin-3a), due to its inability to inhibit the p53-MDMX interaction, leading to an incomplete restoration of p53 activity (Patton et al., 2006; Wade et al., 2006; Graves et al., 2012). Based on this, the effect of OXAZ-1 was tested in human breast cancer MCF-7 cells, a well-known MDMX-overexpressing tumour cell line (Graves et al., 2012). As expected, a similar growth inhibitory effect to that observed in HCT116 p53^{+/+} cells was achieved with OXAZ-1 in MCF-7 cell lines (GI₅₀

value of $23.5 \pm 2.5 \mu\text{M}$). Additionally, as in HCT116 p53^{+/+} cells, also in MCF-7 tumour cells, the OXAZ-1 growth inhibitory effect was associated to a G0/G1-phase cell cycle arrest (Figure 5A) and to a late apoptotic cell death (Figure 5B) with PARP cleavage (Figure 5C). Moreover, also in MCF-7 tumour cells, 23.5 μM 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) (Figure 5D).

Together, these results showed that also in MDMX-overexpression tumour cells OXAZ-1 was able to activate the p53 pathway predominantly inhibited by MDMX.

OXAZ-1 sensitizes tumour cells to chemotherapeutic drugs

It was also investigated if OXAZ-1 increased the sensitivity of tumour cells to the effects of conventional chemotherapeutic drugs such as doxorubicine and etoposide. For that, we investigated the effect of very low concentrations of OXAZ-1 (approximately the GI₅ to GI₁₀ concentration; for which no significant effects on tumour cell growth were observed), on the growth of HCT116 p53^{+/+} cells in combination with increasing concentrations of doxorubicin and etoposide (Figure 6). The results showed that OXAZ-1 significantly increased the sensitivity of tumour cells (from 12% to 19%) at the three concentrations tested of doxorubicine and etoposide.

Analysis of the predicted binding model of OXAZ-1 to MDM2 and MDMX supports that OXAZ-1 binds to both MDM proteins

The OXAZ-1 molecule, presenting the highest percentage of reversion effect on both MDM2 and MDMX, was used in a molecular docking study to shed light on the molecular mode of action. The OXAZ-1 molecule was docked on the MDM2 and MDMX hydrophobic clefts using the Molecular Operating Environment (MOE)

software (version 2013.08) [1] and the crystallographic structures with PDB code 3LBL (MDM2) and 3LBJ (MDMX) allowing ranking the docking poses by binding affinities. On the following discussion, only the top ranked conformation was used.

Recently[artigo **SUBMITTED**] we have re-docked the MI-63 analog co-crystallized MDM2 ligand present in the 3LBL structure with similar docking parameters and found a good root mean square deviation between the predicted and co-crystallized poses (1.11 Å). The WK298 molecule was also re-docked on the MDMX using the 3LBJ structure. It was found that the protocol could also reproduce the crystallographic pose re-creating, this way, the main interactions between the molecule and the protein structure.

MDM2 and MDMX share high sequence homology in their p53 binding domains and interact with three key hydrophobic residues of p53: Phe19, Trp23 and Leu26. Between MDM2 and MDMX, p53 interacts in a similar but not identical hydrophobic cleft with the same three Phe19, Trp23 and Leu26 pockets although for instance, around the latter, some residues are different between both structures [2].

The docking of OXAZ-1 in MDM2 and MDMX led to a similar prediction for the binding energy: -6.76 kcal/mol in MDM2 and -6.80 kcal/mol in MDMX. However, a visual inspection of the binding interactions established between OXAZ-1 with MDM2 and MDMX shows two different binding modes.

Fig. 7 shows the two proteins superposed with the OXAZ-1 top ranked docking poses obtained for each protein. In both poses, the aromatic part of the tosyl moiety is located in the Trp23 pocket, mimicking this hydrophobic interaction. However, the remaining of the molecule interacts with the two proteins in different zones.

In MDM2, the indole moiety of OXAZ-1 makes CH- π interactions with Leu54 and occupies the area reserved for hydrophobic interactions by Leu26 of p53. In MDMX, the MDM2 Phe55 is substituted by an histidine with a different side chain conformation that decreases the size of the cleft near Leu54 and prevents a similar pose as in MDM2. However, this histidine is able to make a CH- π interaction with OXAZ-1 allowing the carbon chain of OXAZ-1, containing the piperidone moiety, to sit on a cleft that runs alongside this residue. The indole moiety in this pose is located in the region defined by the p53 Phe19 residue.

Discussion

MDM2 and MDMX are two major factors that contribute to cancer development due to the inactivation of wt p53. Disruption of the p53-MDMs regulatory network has therefore clear implications for tumorigenesis and presents exciting opportunities for cancer therapy (Wade et al., 2013). The antitumor activity of small molecule inhibitors of the p53-MDMs interaction has already been confirmed in preclinical studies and early-phase of clinical trials. However, to date, most of the current efforts have been focused on the p53-MDM2 interaction (Li and Lozano, 2013; Wade et al., 2013). 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 (Li and Lozano, 2013; Graves et al., 2012). This clearly indicates that dual inhibition of MDM2 and MDMX may substantially improve the outcome of this p53 activation strategy due to the full p53 activation (Li and Lozano, 2013; Wade et al., 2013). In spite of this, recent efforts to develop this type of molecules have been hindered by structural differences in the p53 pockets of MDM2 and MDMX (Riedinger and McDonnell, 2009). In fact, only recently, the small molecule RO-5963 was identified as the first dual inhibitor of MDM2 and MDMX, highly effective against tumour cells with high levels of MDMX (Graves et al., 2012).

In this work, a yeast target-directed assay was used for the screening of inhibitors of the p53-MDM2/X interaction. From the analysis of a small library of tryptophanol-derived oxazolopiperidone lactams, the *N*-tosylindole OXAZ-1 was identified as a potential dual inhibitor of the p53 interaction with MDM2 and MDMX. The assessment of the predicted binding model of OXAZ-1 to MDM2 and MDMX, by

computational docking, supported the results from yeast, showing that OXAZ-1 is capable to bind to both MDM2 and MDMX.

The analysis of the *in vitro* antitumor activity revealed that the tumour growth inhibitory effect of OXAZ-1 was highly dependent on p53. Moreover, in conformity with the results from yeast, OXAZ-1 exhibited typical hallmarks of a dual inhibitor of MDM2 and MDMX (Li and Lozano, 2013). Particularly, it decreased the proliferation of wt p53-carrying tumour cells, an effect associated with the induction of cell cycle arrest and apoptosis. Additionally, it led to the stabilization of the p53 protein levels, what indicates an inhibition of the MDM2-mediated p53 degradation by OXAZ-1, and up-regulation of the p53 transcriptional activity increasing the protein levels of the p53 target genes MDM2, MDMX, p21, Bax and Puma. Finally, it presented reduced antitumor activity against tumours without p53. Beyond that, like RO-5963, OXAZ-1 efficiently reduced the proliferation of wt p53-carrying tumour cells with high levels of MDMX (MCF-7 cells). In fact, also in MDMX-overexpression tumour cells, OXAZ-1 induced cell cycle arrest and apoptosis, led to p53 stabilization, and up-regulated p53 transcription targets involved in cell cycle and apoptosis. OXAZ-1 therefore overcame the commonly reported (Patton et al., 2006; Wade et al., 2006; Graves et al., 2012) resistance of MDMX-overexpression tumour to MDM2-only inhibitors.

Cancer cell mitochondria are structurally and functionally different from their normal counterparts. Additionally, tumour cells are more susceptible to mitochondrial perturbations than the normal cells. Based on this, mitochondrially-targeted agents have emerged as a promising approach to selectively eradicate chemotherapy-refractory cancer cells (Fulda et al., 2010). In fact, the (re)activation of cell death programmes by pharmacological agents that induce or facilitate mitochondrial membrane permeabilization (MMP) have emerged as an attractive strategy for cancer treatment

(Fulda et al., 2010). MMP can be triggered by agents that increase cytosolic calcium or stimulate ROS generation. Moreover, MMP can be favoured 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 (Chipuk et al., 2004; Fulda et al., 2010). MMP results in the immediate $\Delta\psi_m$ dissipation, with the consequent release of pro-apoptotic factors into the cytosol, such as cyt *c* (Fulda et al., 2010). The results obtained in the present work showed that OXAZ-1 also targets mitochondria of tumour cells. Actually, OXAZ-1 potently triggered a mitochondrion-centered apoptotic cell death characterized by ROS generation, Bax translocation to mitochondria, $\Delta\psi_m$ dissipation, and the subsequent mitochondrial cyt *c* release.

The pharmacological activation of the p53 pathway can be exploited to work in combination with other therapeutic agents to promote cell death via p53-dependent and -independent mechanisms. It is still not completely understood how drug combinations with p53 activators can lead to synergistic enhancement of cell death and improved therapeutic efficacy in the treatment of cancer (Hoe et al., 2014). 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, such as mitotic inhibitors, CDK inhibitors, DNA-damaging agents, and radiation therapy (Wade et al., 2013; Hoe et al., 2014). Based on this, the potential antitumor activity of OXAZ-1 is therefore strengthened in this work by showing that this compound may prime tumour cells for death induced by chemotherapeutic drugs, such as etoposide and doxorubicin. The prospect of combining conventional chemotherapeutic agents with OXAZ-1 represents a promising strategy to minimize the emergence of resistance and to achieve maximal therapeutic responses with minimal side effects in cancer therapy.

In summary, several evidence are provided in this work for the significant potential of OXAZ-1 as an anticancer agent. The *in vitro* antitumor activity of OXAZ-1 is attributable to the selective activation of the p53 pathway involving the potential dual inhibition of MDM2 and MDMX. Additionally, OXAZ-1 leads to the (re)activation of a mitochondria-mediated cell death programme, being highly effective in combination with conventional therapies by increasing the antitumor outcome at very low concentrations.

Further work is still required to completely clarify the molecular mechanism of action of *N*-tosylindole OXAZ-1. In spite of this, this study opens the way to a new class of 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 *N*-tosylindole OXAZ-1 may be the first step toward the development of promising dual inhibitors of MDM2 and MDMX.

Acknowledgments

This work received the financial support from the European Union (FEDER funds through COMPETE) and National Funds (FCT, Fundação para a Ciência e Tecnologia) through REQUIMTE (Pest-C/EQB/LA0006/2013) and iMed.Ulisboa (Pest-OE/SAU/UI4013/2014), and through the research projects PTDC/QUI-QUI/111664/2009, and PTDC/SAU-FAR/110848/2009 (FCOMP-01-0124-FEDER-015752). It was also supported by 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).

Figure Legends

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

Figure 2. Identification of OXAZ-1 as potential dual inhibitor of the p53 interaction with MDM2 and MDMX, using a yeast screening assay. (A) Schematic representation of the yeast target-directed screening assay. Human MDM2 and MDMX block the growth inhibition and the increase of actin expression levels induced by human wt p53 due to the inhibition of p53 transcriptional activity. Nutlin-3a (a known inhibitor of the p53-MDM2 interaction) and SJ-172550 (a known inhibitor of the p53-MDMX interaction) revert the MDM2 and MDMX inhibitory effects, respectively, on p53, re-establishing the p53 activity. (B) Effect of 0.1 – 50 μ M of OXAZ-1, nutlin-3a and SJ-172550 on the inhibition of p53-induced yeast growth arrest by MDM2 and MDMX, after 42 h incubation. The growth of yeast cells co-expressing p53 with MDM2 or 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 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (C) Effect of 0.1 – 50 μ M OXAZ-1 on the growth of yeast cells expressing p53 alone and control yeast 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 ($p > 0.05$). (D) Effect of 10 μ M OXAZ-1 on the actin protein levels of yeast cells co-expressing p53 with MDM2 or MDMX after 42 h incubation. Immunoblots represent

one of two independent experiments; Pgk1p was used as loading control; band intensities were normalized against the control sample (DMSO), which was set as 1.

Figure 3 – OXAZ-1 exhibits *in vitro* antitumor activity through selective activation of the p53 pathway in human colon adenocarcinoma HCT116 tumour cells. (A)

The GI₅₀ concentration of OXAZ-1 was determined in p53^{+/+} and p53^{-/-} HCT116 cells, after 48 h incubation, 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)** OXAZ-1 induces G0/G1-phase cell cycle arrest in HCT116 p53^{+/+} cells.

The effect was determined after 24 h treatment with 25 μM (GI₅₀) and 50 μM (2xGI₅₀) of OXAZ-1; cell cycle phases were analysed by flow cytometry using PI and quantified using ModFit LT software; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated (***p* < 0.01, ****p* < 0.001). **(C)**

OXAZ-1 induces late apoptosis in HCT116 p53^{+/+} cells. The effect was determined after 24 h treatment with 25 μM (GI₅₀) and 50 μM (2xGI₅₀) of OXAZ-1. Apoptosis was analysed by flow cytometry using FITC-Annexin V and PI; data are mean ± S.E.M. of two independent experiments; values significantly different from DMSO are indicated

(****p* < 0.001). **(D, E)** OXAZ-1 leads to PARP cleavage **(D)**, and increases the protein levels of p53, MDM2, MDMX, p21, Puma and Bax **(E)** in p53^{+/+}, but not in p53^{-/-}, HCT116 cells. Western blot analysis was performed after 24 h treatments with 25 μM OXAZ-1 or DMSO only; immunoblots represent one of two independent experiments;

GAPDH was used as loading control; band intensities were normalized against the control sample (DMSO), which was set as 1.

Figure 4. OXAZ-1 induces a mitochondrial-dependent apoptotic pathway in HCT116 p53^{+/+} tumour cells. (A) HCT116 p53^{+/+} cells were treated for 8 h (in $\Delta\psi_m$ analysis) or for 48 h (in analysis of ROS production) with 25 μ M OXAZ-1 or DMSO only. For assessment of ROS production and $\Delta\psi_m$, cells were stained with CellROX and DiOC₆(3), respectively, and analysed by flow cytometry; histograms represent one of two independent experiments; M2 cursor indicates the subpopulation analysed; values correspond to the increase in the percentage of CellROX positive cells (in analysis of ROS production) and of cells with $\Delta\psi_m$ dissipation obtained after treatment with OXAZ-1 and are mean \pm S.E.M. of two independent experiments. In the $\Delta\psi_m$ analysis, FCCP was used as positive control. (B) 25 μ M OXAZ-1 induces translocation of Bax from cytosol to mitochondria. (C) 25 μ M OXAZ-1 triggers mitochondrial cyt *c* release into the cytosol after 24 h treatment. Immunoblots represent one of two independent experiments; GAPDH and COX-IV were used as loading controls of cytosolic and mitochondrial fractions, respectively.

Figure 5. OXAZ-1 exhibits antitumor activity against MDMX-overexpressing human breast adenocarcinoma MCF-7 tumour cells through activation of the p53 pathway. (A) OXAZ-1 induces G0/G1-phase cell cycle arrest in MCF-7 cells. The effect was determined after 24 h treatment with the 2xGI₅₀ (47 μ M) of OXAZ-1; cell cycle phases were analysed by flow cytometry using PI; data are mean \pm S.E.M. of two independent experiments; values significantly different from DMSO are indicated (** $p < 0.01$). (B) OXAZ-1 induces late apoptosis in MCF-7 cells. The effect was determined after 24 h treatment with the 2xGI₅₀ concentration of OXAZ-1. Apoptosis was analysed 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) OXAZ-1 leads to PARP cleavage (C), and increases the protein levels of p53, p21 and Bax (C) in MCF-7 tumour cells. Western blot analysis was performed after 24 h treatments with 23.5 μ M OXAZ-1 or DMSO only; immunoblots represent one of two independent experiments; GAPDH was used as loading control; band intensities were normalized against the control sample (DMSO), which was set as 1.

Figure 6. OXAZ-1 sensitizes tumour cells to the effects of etoposide and doxorubicin. The effect of the compounds on cell growth was analysed following 48 h incubation, using the SRB assay. HCT116 p53^{+/+} tumour cells were treated with increasing concentrations of doxorubicin (9.38 to 37.5 nM) or etoposide (0.38 to 3.00 μ M) in the presence of a very low concentration (between GI₅ to GI₁₀) of OXAZ-1 or DMSO only. Data are mean \pm S.E.M. of three to four independent experiments. Values significantly different from DMSO are indicated (** $p < 0.01$; *** $p < 0.001$).

Figure 7. Docking pose of OXAZ-1 within the MDM2 and MDMX hydrophobic clefts limits depicted with a surface (in green, hydrophobic; in pink, hydrophilic areas). LEU54 and ILE61 are highlighted and CH- π interactions are displayed through dotted lines. The OXAZ-1 pose found with the MDM2 structure is represented with carbon atoms in orange and the one for MDMX is represented with carbon atoms in blue.

Figure 8. Proposed mechanism of action of OXAZ-1. OXAZ-1, as putative dual inhibitor of the p53 interaction with MDM2 and MDMX, leads to p53 stabilization and to the subsequent activation of p53 transcriptional activity with increased levels of p53 target proteins, as 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 production, $\Delta\Psi_m$ dissipation, and cyt c release. The release of cyt c from mitochondria triggers the activation of a caspase pathway with PARP cleavage; *p53 target genes.

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 with MDM2 or 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 or MDMX inhibitory effects obtained for 0.1 - 50 μM compound (see Figure 2B for OXAZ-1 and positive controls). Data are mean ± S.E.M. of 6 independent experiments.

References

Phillips, A.; Teunisse, A.; Lam, S.; Lodder, K.; Darley, M.; Emaduddin, M.; Wolf, A.; Richter, J.; Lange, J.; Verlaan-de Vries, M.; Lenos, K.; Bohnke, A.; Bartel, F.; Blaydes, J.P.; Jochemsen, A.G. HDMX-L is expressed from a functional p53-responsive promoter in the first intron of the HDMX gene and participates in an autoregulatory feedback loop to control p53 activity. *J Biol Chem.*, **2010**, 285, 29111-29127.

Li, B.; Cheng, Q.; Li, Z.; Chen, J. p53 inactivation by MDM2 and MDMX negative feedback loops in testicular germ cell tumors. *Cell Cycle*, **2010**, 9, 1411-1420.

Vassilev, L.T.; Vu, B.T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E.A. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*, **2004**, 6, 844-848.

Reed, D.; Shen, Y.; Shelat, A.A.; Arnold, L.A.; Ferreira, A.M.; Zhu, F.; Mills, N.; Smithson, D.C.; Regni, C.A.; Bashford, D.; Cicero, S.A.; Schulman, B.A.; Jochemsen, A.G.; Guy, R.K.; Dyer, M.A. Identification and Characterization of the First Small Molecule Inhibitor of MDMX. *Journal of Biological Chemistry*, **2010**, 285, 10786-10796.

Graves, B., Thompson, T.; Xia, M.; Janson, C.; Lukacs, C.; Deo, D.; Di Lello, P.; Fry, D.; Garvie, C.; Huang, K.S.; Gao, L.; Tovar, C.; Lovey, A.; Wanner, J.; Vassilev, L.T. Activation of the p53 pathway by small-molecule-induced MDM2 and MDMX dimerization. *Proceedings of the National Academy of Sciences of the United States of America*, **2012**, 109, 11788-11793.

Wade, M.; Li, Y.C.; Wahl, G.M. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nature Reviews Cancer*, **2013**, 13, 83-96.

Hoe, K.K.; Verma, C.S.; Lane, D.P. Drugging the p53 pathway: understanding the route to clinical efficacy. *Nature Reviews Drug discovery*, **2014**, 13, 217-236.

Li, Q.; Lozano, G. Molecular pathways: targeting Mdm2 and Mdm4 in cancer therapy. *Clin Cancer Res.*, **2013**, 19(1), 34-41.

Patton, J.T.; Mayo, L.D.; Singhi, A.D.; Gudkov, A.V.; Stark, G.R.; Jackson, M.W. Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. *Cancer Res.*, **2006**, 66(6), 3169-76.

Wade, M.; Wong, E.T.; Tang, M.; Stommel, J.M.; Wahl, G.M. Hdmx modulates the outcome of p53 activation in human tumor cells. *J Biol Chem.*, **2006**, 281(44), 33036-44.

Riedinger, C.; McDonnell, J.M. Inhibitors of MDM2 and MDMX: a structural perspective. *Future Med Chem.*, **2009**, 1(6), 1075-94.

Chipuk, J.E.; Kuwana, T.; Bouchier-Hayes, L.; Droin, N.M.; Newmeyer, D.D.; Schuler, M.; Green, D.R. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*, **2004**, 303(5660), 1010-4.

Fulda, S.; Gorman, A.G.; Hori, O.; Samali, A. Cellular Stress Responses: Cell Survival and Cell Death. *International Journal of Cell Biology*, **2010**

Leão, M.; Pereira, C.; Bisio, A.; Ciribilli, Y.; Paiva, A.; Machado, N.; Palmeira, A.; Fernandes, M.X.; Sousa, E.; Pinto, M.; Inga, A.; Saraiva, L. Discovery of a new small-molecule inhibitor of p53-MDM2 interaction using a yeast-based approach. *Biochemical Pharmacology*, **2013a**, 85, 1234-1245.

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 Journal*, **2013b**, 280, 6498-507.

Mir, R.; Tortosa, A.; Martinez-Soler, F.; Vidal, A.; Condom, E.; Perez-Perarnau, A.; Ruiz-Larroya, T.; Gil, J.; Gimenez-Bonafe, P. Mdm2 antagonists induce apoptosis and synergize with cisplatin overcoming chemoresistance in TP53 wild-type ovarian cancer cells. *Int J Cancer*, **2013**, 132(7), 1525-36.

Ray-Coquard, I.; Blay, J-Y.; Italiano, A.; Le Cesne, A.; Penel, N.; Zhi, J.; Heil, F.; Rueger, R.; Graves, B.; Ding, M.; Geho, D.; Middleton, S.A.; Vassilev, L.T.; Nichols, J.L.; Bui, B.N. Effect of the MDM2 antagonist RG7112 on the P53 pathway in patients with MDM2-amplified, well-differentiated or dedifferentiated liposarcoma: an exploratory proof-of-mechanism study. *The Lancet Oncology*, **2012**, 13(11), 1133-1140.

Pereira, N.A.L.; Sureda, F.X.; Espuglas, R.; Pérez, M.; Amat, M.; Santos, M.M.M. Tryptophanol-derived oxazolopiperidone lactams: identification of a hit compound as NMDA receptor antagonist. *Bioorg. Med. Chem. Lett.*, **2014**, 24, 3333-3336.

Amat, M.; Santos, M.M.M.; Bassas, O.; Llor, N.; Escolano, C.; Gómez-Esqué, A.; Molins, E.; Allin, S.M.; McKee, V., Bosch, J. Straightforward methodology for the enantioselective synthesis of benzo[a]- and indolo[2,3-a]quinolizidines. *J. Org. Chem.*, **2007**, 72 (14), 5193-5201.

Amat, M.; Santos, M.M.M.; Gómez, A.M.; Jokic, D.; Molins, E.; Bosch, J. Enantioselective spirocyclizations from tryptophanol-derived oxazolopiperidone lactams. *Org. Lett.*, **2007**, 9(15), 2907-2910.

[1] Molecular Operating Environment (MOE), v2013.08, Chemical Computing Group: Montreal, 2013.

[3] Y. Zhao, D. Bernard and S. Wang, “Small Molecule inhibitors of MDM2-p53 and MDMX-p53 interaction as new cancer therapeutics”, *BioDiscovery* 2013; 8: 4; DOI: 10.7750/BioDiscovery.2013.8.4