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3-Arylidene-N-hydroxyoxindoles: a new class of compounds endowed with antitumor activity

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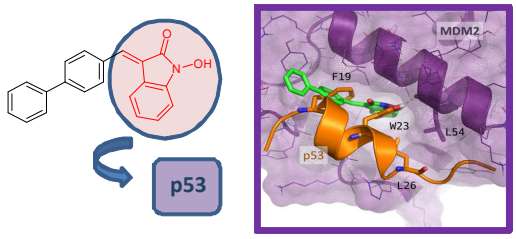

Exploring new scaffolds: A series of 3-arylidene-N-hydroxyoxindoles showed potent antiproliferative and proapoptotic activity against wild-type p53 IGROV-1 ovarian carcinoma cell line and a considerably lower efficacy against the mutant IGROV-1/Pt1 subline lacking p53 function.. The results support a role of this transcription factor as a determinant of cytotoxicity. Treatment of an IGROV-1 xenograft growing as ascitic tumor produced appreciable increase of survival of tumor -bearing animals.

3-Arylidene-N-hydroxyoxindoles: a new class of compounds endowed with antitumor activity

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Abstract

A series of compounds containing the N-hydroxyoxindole scaffold were synthesized and evaluated for their antitumor activity. The compounds showed a potent antiproliferative activity against wild-type p53 IGROV-1 ovarian carcinoma cell line and a considerably lower efficacy against the mutant IGROV-1/Pt1 subline lacking p53 function. The differential response of ovarian carcinoma cells depending on p53 status was also reflected in the different susceptibility to apoptosis of the treated cell lines. The results support a role of this transcription factor as a determinant of cytotoxicity.

The therapeutic potential of the most representative compound of the series was evaluated in the treatment of an IGROV-1 xenograft growing as ascitic tumor. Using i.p. administration, daily treatment with the compound for 3 weeks produced a significant increase of survival of tumor-bearing animals.

The easily available 3-(hetero)arylidene-oxindoles[1] are a privileged scaffold for compounds endowed with a variety of biological properties, mainly antitumor, but also antibacterial,[2,3] antifungal,[2] cardiotonic[4] and antioxidative.[5] The antitumor activity appears to be mediated by different mechanisms, mainly inhibition of kinases. [6-13]

A very significant result of these studies was the development of Sunitinib malate (Sutent®, Pfizer) [14], an inhibitor of receptor tyrosine kinases, which was approved in 2006 for the treatment of gastrointestinal stromal tumours and advanced renal-cell carcinoma.

Differently from the oxindoles, the corresponding N-hydroxy analogues have been considerably less studied. To the best of our knowledge, only a few examples of biologically active N-hydroxy oxindoles have been reported in the literature so far. Compounds containing this scaffold were studied as influenza virus[15] and Ret tyrosine kinase [12] inhibitors as well as for the treatment of Multiple Sclerosis.[16]

As part of a previous research program aimed at studying new HDAC inhibitors, we have developed a series of hydroxamic acid-based compounds, characterized by a cinnamic spacer capped with a substituted phenyl group (**1**, Figure 1).[17] Recently, we have initiated the search for ZBG (Zinc binding group)-functionalities that could replace the hydroxamic acid group[18]

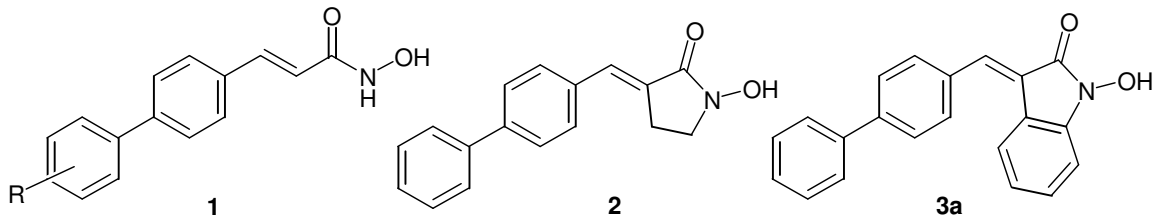


Figure 1. General structure of HDAC inhibitors **1** containing the 4-vinylbiphenyl scaffold and structures of compounds **2** and **3a**.

Among the analogues incorporating alternative ZBGs, we synthesized compound **2** containing a 5-membered cyclic hydroxamic acid.[18]

Unfortunately, compound **2** showed a lack of activity in terms of both HDAC inhibition (IC_{50} >5 μM on HDAC-2 from HeLa cells) and cell growth inhibition (IC_{50}>20 μM against a panel of tumor cell lines). Therefore, we replaced the hydroxypyrrolidinone moiety with a N-hydroxyoxindole counterpart (compound **3a**). While showing a very low activity on the enzyme (IC_{50} >5 μM on HDAC-2 from HeLa cells) as much as **2**, the new derivative **3a** had a potent cell growth inhibitory activity on IGROV-1 (IC_{50} 0.1±0.05 μM) and HCT 116 (IC_{50} 1.3±0.1 μM) tumor cell lines. We speculated that this activity could be due to the N-hydroxyoxindole core, thus we decided to investigate this promising and almost unstudied scaffold. A series of analogues containing the N-hydroxyoxindole skeleton were synthesized and their biological activity was evaluated. Here we show the results of this preliminary exploration. Compounds **3a-g** were prepared by Knoevenagel condensation onto 1-hydroxy-1,3-dihydroindol-2-one **5**, in turn obtained from methyl 2-

nitrophenylacetic acid[19] by reduction with Zn/NH_4Cl.[20] Compound **3h** was obtained by methylation of compound **3a**. (Scheme 1).

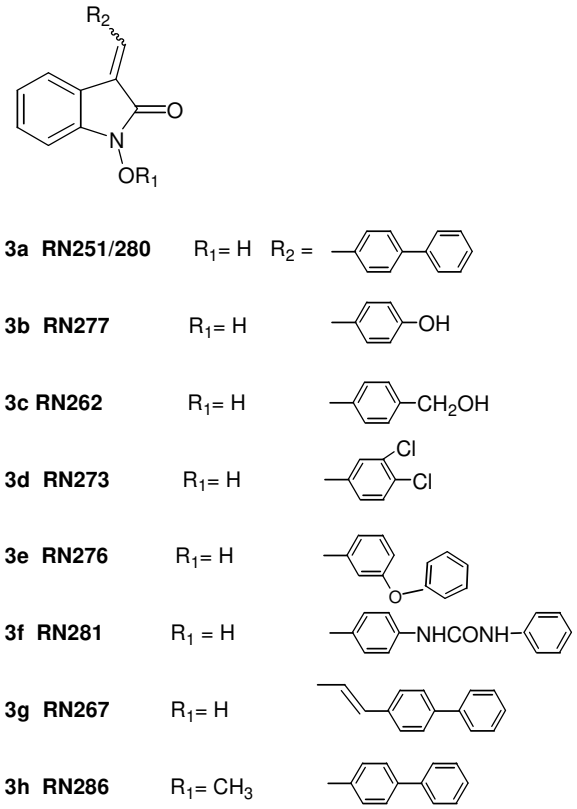
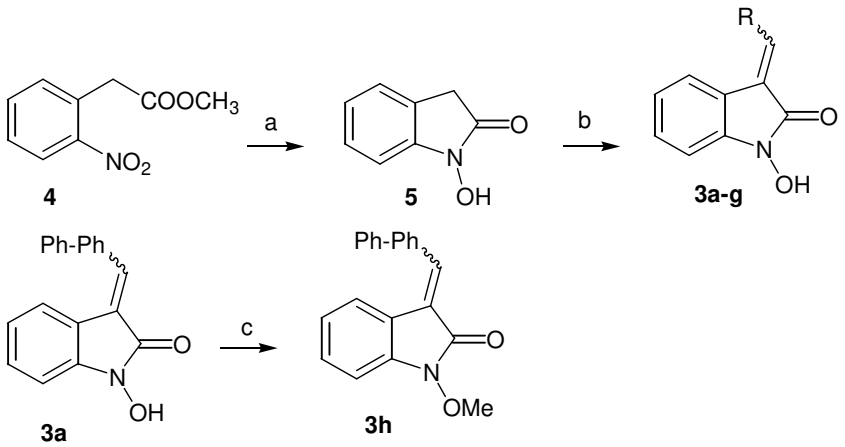


Figure 2. Structures of compounds **3a-h**



Scheme 1. Synthesis of compounds **3a-h**. Reagents and conditions: a) Zn, NH_4Cl, CH_3OH, H_2O, rt, 1.5h, 50%; b) RCHO, EtOH, piperidine, reflux, 1-5h, 30-85%; c) K_2CO_3, CH_3CN, methyl iodide, reflux, 6 h, 58%.

The N-hydroxyoxindoles **3a-h** were obtained as mixtures of E/Z isomers.

NOE experiments on a series of 3-arylideneoxindoles demonstrated that the chemical shifts of the *ortho* protons of the benzylidene ring were around 7.8-8.5 ppm for the *Z* isomer and 7.4-7.8 ppm for the *E* isomer.[21] Analogously, to determine the *E/Z* stereochemistry of compounds **3** we conducted NOE experiments on compound **3a** (see SI), confirming that the *ortho* protons of the benzylidene ring in the Z isomer are deshielded relative to those of the *E* isomer. Thus, the relative ratios of the *E* and *Z* stereoisomers within this N-hydroxyoxindole series (compounds **3a-f**, **3h**) was assigned based on the chemical shifts of protons at the C-2' and C-6' positions. On the contrary, the configuration of compound **3g** was assigned based on the chemical shift of proton H-2' on the phenylallylidene moiety. [22]

Previous investigations on 3-arylideneoxindoles have reported the *E/Z* isomerization to be solvent, temperature, time and light dependent. [21-24] To determine the stability of our compounds over time, we separated the two isomers of **3a** by flash column chromatography. The isomerization was studied by ^{1}H NMR spectroscopy, as this method can be carried out quantitatively in real-time. Freshly prepared solutions of both isomers in DMSO-d_6 were monitored at various time intervals, evaluating the chemical shift of the benzylidene ring *ortho* protons. The results evidenced that equilibration occurred within 3 h. Thus, it is reasonable to assume that the N-hydroxyoxindoles, prepared in DMSO stock solution, would be mixtures of E/Z isomers when tested in biological assays.

The prepared compounds were tested for antiproliferative activity against IGROV-1 and its subline IGROV-1/Pt1 (human ovarian carcinoma cell lines) selected for resistance to Cisplatin (Table 1), the former having functional p53, whereas the latter is p53-defective. N-hydroxyindolinone **3a** exhibited a potent antiproliferative activity against IGROV-1 cells and a considerably lower inhibitory activity against the mutant IGROV-1/Pt1 subline lacking p53 function.

Table 1. Antiproliferative activity (IC_{50}, μM) of compounds **3a-h** against IGROV-1 and IGROV-1/Pt1 cell lines

Cpd	**IC_{50} (μM)**		***E:Z***
	IGROV-1	IGROV-1/Pt1	
3a	0.1±0.1	6.6±1	8:2
3b	2.62±0.49	38.95±1.63	8:2
3c	12.7±3.5	36.8±3.5	9:1
3d	0.23	5.6±0.7	8:2
3e	7.8±1.8	20.3	9:1

3f	2.4±0.6	12.9±1.1	5:95
3g	3.7±1.1	12.2±4.3	5:95[a]
3h	1.65±0.9	15.05	95:5

[a] ratio *ZE: EE* 95:5 [22]

All the analogues **3b-g** exhibited a significant antiproliferative activity against IGROV-1 cells with IC_{50} values in the micromolar range (Table 1). The introduction of a N-methoxy group in place of the N-hydroxy group caused a decrease of the activity (**3h** vs **3a**). A common feature of the compounds of this series was a differential inhibitory activity against IGROV1/Pt1 subline lacking p53 function (Table 1). Compound **3a**, the most active of the series, was chosen to further investigate this feature. The compound was tested on A431 cells (from a squamous cell carcinoma) characterized by p53 mutation. The reduced sensitivity of these cells to **3a** was consistent with the influence of the p53 function on the cytotoxic activity. (Figure 3).

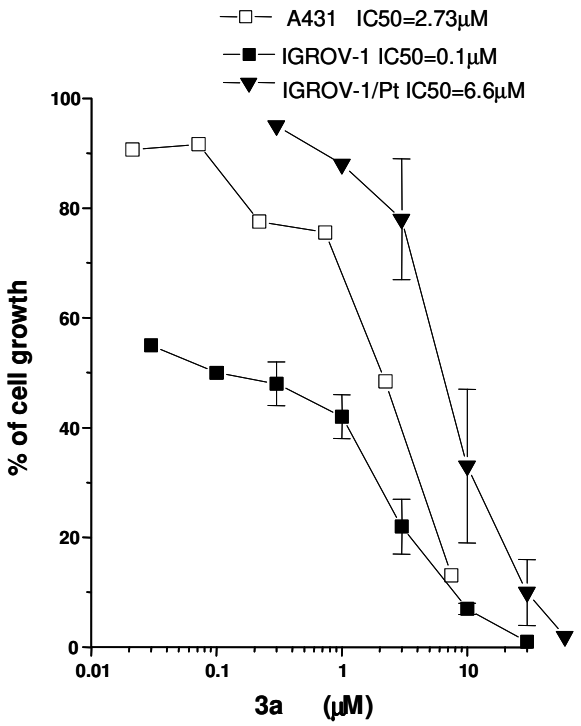


Figure 3. Dose-response curves for IGROV-1, IGROV-1/Pt1 and A431 tumor cell lines treated for 72h with compound **3a**.

The differential response of the two ovarian carcinoma cell lines was also reflected in the different susceptibility to apoptosis induced by cytotoxic concentrations (IC_{80}) of **3a** (Figure 4).

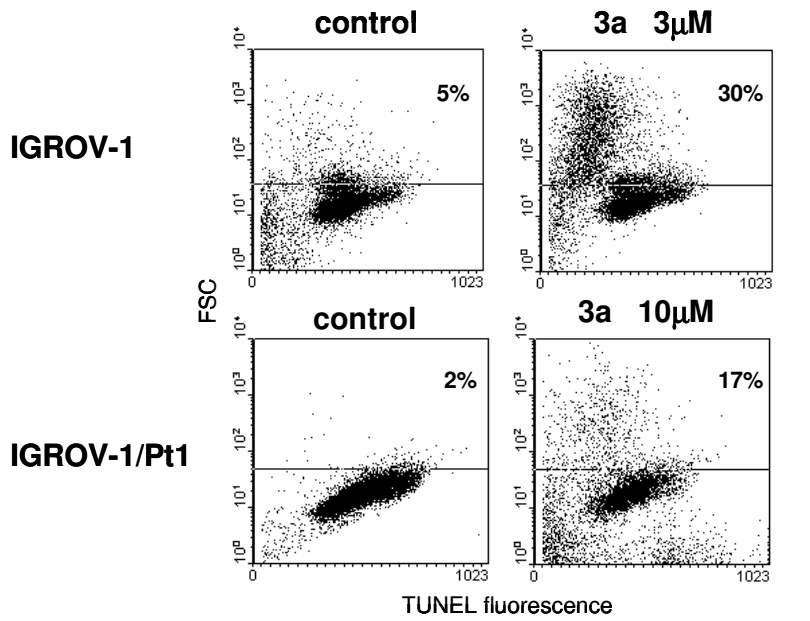


Figure 4. Flow cytometric analysis of apoptosis induced after the exposure to compound **3a** (IC_{80} value) in ovarian carcinoma cells IGROV-1 and ovarian carcinoma cells IGROV-1/Pt1. The extent of apoptosis was determined 72 h after drug exposure by TUNEL assay.

The wild type p53cells were more susceptible to apoptosis induction (apoptosis level about 30% after 72 h exposure) than the p53 mutant cells (apoptosis level about 17%) in spite of exposure to high concentration (10 μM). The ability to induce apoptosis was also consistent with the substantial increase of sub-G1 function of treated cells (Figure 5). Following a short term (4 h) exposure, compound **3a** was able to induce p53 activation, as detected by p53 protein phosphorylation and acetylation (Figure 5).

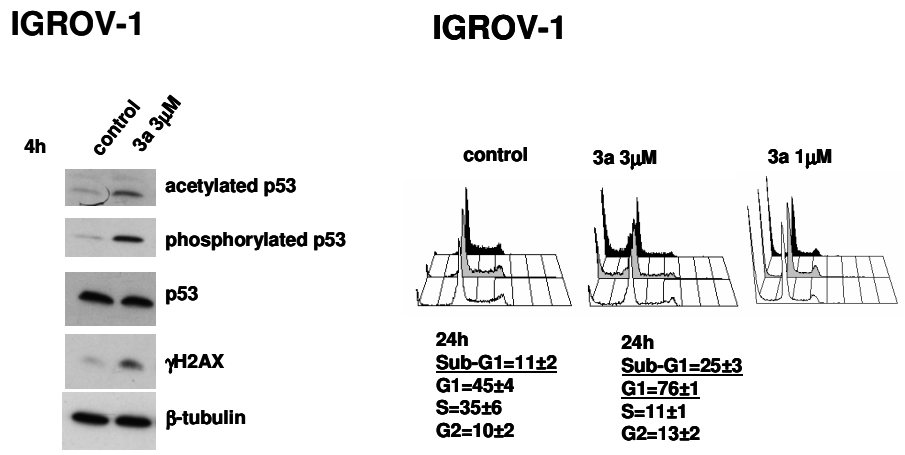


Figure 5. a) Effects of compound **3a** on acetylation, phosphorylation of p53 and activation of histone γH2AX in IGROV-1 cells. Cells were treated for 4h with cytotoxic concentration (3μM, IC_{80}) of compound **3a** . Cell lysates were prepared and examined by western-blot analysis. The blots were reprobed with β-tubulin as a loading control. b) Cell

cycle perturbation induced after the exposure to compound **3a** (IC_{80} and IC_{50} value) in ovarian carcinoma cells IGROV-1.. Time course analysis of cell cycle was performed by propidium-iodide staining.

All these results support a role of p53 in response to treatment with N-hydroxyoxindoles.

A number of compounds are known to enhance the function of wild-type p53 by increasing its stability through various mechanisms.[25] Most of them (nutlins, benzodiazepinediones and spiro-oxindoles) act by targeting the MDM2:p53 interaction. Noteworthy, a recent work has demonstrated that 3-arylideneoxindoles inhibit the MDM2-p53 interaction.[26] All these considerations led us to speculate that 3-arylidene-N-hydroxyindolin-2-ones could interact with the p53 binding site on MDM2.

MDM2 protein has been extensively studied in more than 30 high resolution X-ray and nuclear magnetic resonance (NMR) structures with and without p53-derived peptides and small molecules. Analysis of all available complexes showed the p53 binding site to be relatively unchanging, displaying no major induced fit. MDM2 shows on its surface a deep and structured binding site for p53, mainly constituted by three hydrophobic pockets occupied by the “hot-spot triad” made up by p53's W23, L26 and F19 (Figure 6A). All three hydrophobic amino acids undergo multiple van der Waals contacts with the surrounding MDM2's residues, while W23 forms an additional hydrogen bond with the Leu54 backbone carbonyl group.

In order to explain the biological activity of compounds **3a-h** and to investigate the possible interaction of such compounds with the p53 binding site on MDM2, molecular docking studies have been performed. In details, the X-ray crystal structure of the 109-residue amino-terminal domain of MDM2 in complex with the 15-residue transactivation domain peptide of p53 has been retrieved from RCSB Protein Data Bank (PDB code 1YCR).[27] p53 fragment was removed from the complex and the MDM N-terminal region was prepared for the docking procedure by using the Protein Preparation Wizard[28] protocol implemented in Maestro.[29] All compounds were drawn and prepared by using the LigPrep module.[30] For all the compounds both the Z and E isomers were considered. Docking studies were performed by using Glide[31, 32] software.

Our results show that N-hydroxyoxindoles interact with the p53 cleft on MDM2 with a reasonable binding mode. In Figure 6B the best docking pose found for the most active compound **3a** has been reported. The oxindole moiety occupies the W23 pocket on MDM2, while the R2 substitution partially overlaps the position of F19. A hydrogen bond occurs between the NH group of the core scaffold and the carbonyl oxygen of L54, resembling the hydrogen bond found between W23 from p53 and L54.

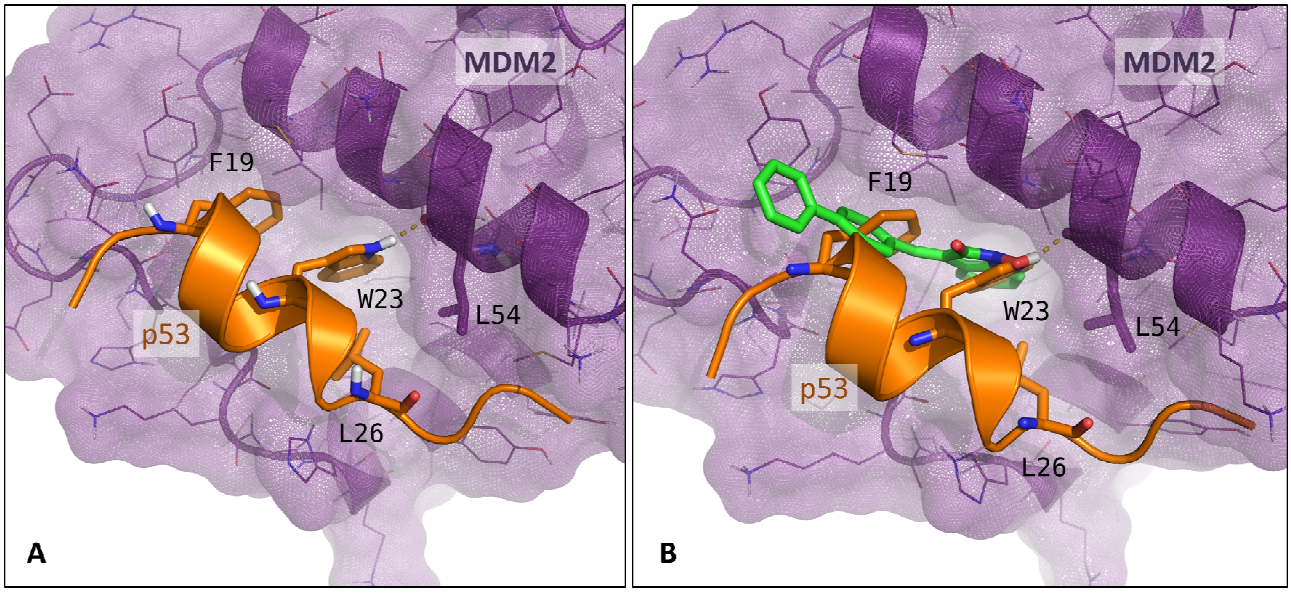


Figure 6. A) Molecular interactions between p53 (orange cartoon) and MDM2 (violet cartoon). L54 has been shown in sticks. Hydrogen bonds are represented as yellow dashes. B) Superimposition between p53 and the docking pose of compound **3a**. The hydrogen bond between **3a** and the backbone carbonyl group of L54 is represented as yellow dashes.

Compound **3a** was chosen to evaluate the therapeutic potential in the treatment of IGROV-1 xenograft growing as ascitic tumor. Using i.p. administration, daily treatment with **3a** (10 mg/kg, qd x 5 day/week) for 3 weeks, produced an appreciable increase (40%) of survival of tumor-bearing animals (data not shown). The efficacy of the treatment was also evidenced by a delay in ascites onset (Figure 7).

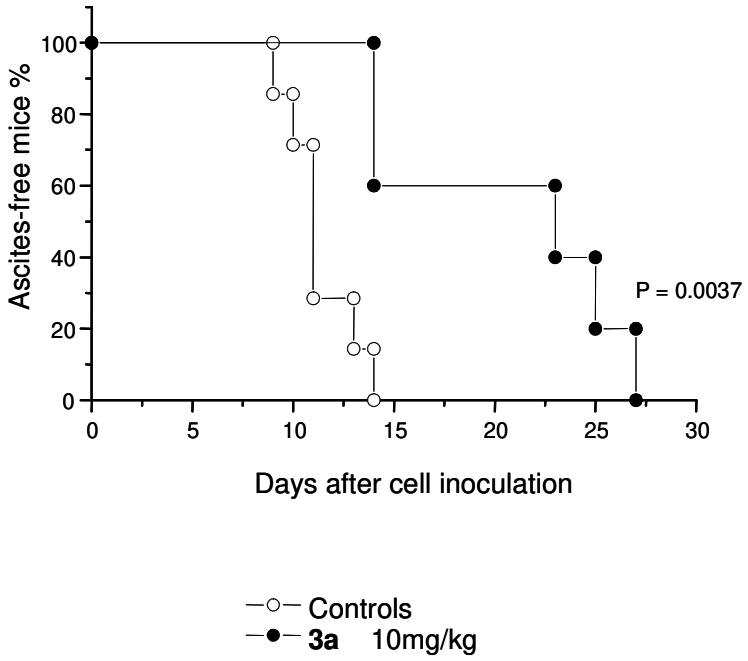


Figure 7. Disease onset in athymic nude mice bearing ascitic ovarian carcinoma IGROV-1 treated i.p. with **3a** (10 mg/kg, qd x 5 day/week) for 3 weeks.

In conclusion, a series of compounds containing the N-hydroxyoxindole scaffold showed a potent antiproliferative activity against IGROV-1 cells and a considerably lower inhibitory activity against the mutant IGROV-1/Pt1 subline lacking p53 function. Biochemical analysis of cells treated with the most active compound did not detect appreciable modifications of H3 or H4 histones or tubulin acetylation. However, the results indicated activation of p53 and apoptosis induction in response to drug treatment. It is well known that the functional wild-type p53 plays a role in the control of tumor growth. Efficacy of antitumor therapy often correlates with cell ability to activate p53 dependent apoptosis. In contrast, loss of p53 function may confer resistance to various antitumor agents. Although the molecular pathways involved in modulation of p53 remain to be defined, optimization of the molecules described in our study may have therapeutic implications. Further biological tests and SAR studies on this new series of compounds are currently underway, in the perspective of a potential development.

Acknowledgments

This work was performed within the framework of COST Actions CM1106 (Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells)

Experimental section

The synthesis of all compounds together with the biological experimental procedures are described in detail in the Supporting Information.

Keywords: N-hydroxyoxindole, antiproliferative activity, p53, apoptosis, antitumor agents.

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