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Article

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Synthesis and antineoplastic evaluation of novel unsymmetrical 1,3,4-oxadiazoles.

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ABSTRACT

A series of novel 1,3,4-oxadiazoles was synthesized and evaluated for their cytotoxic activity in *in vitro* tumor models. Four of the new compounds (**2d**, **2j**, **2k** and **2n**) showed growth inhibition in the XTT dye assay. The most active agent, **2j** showed high potency against human cancer cells with IC_{50} ranging from 0.05 to 1.7 μM. Preliminary SAR correlations suggested that the nature of chains on the oxadiazole is important for antitumor potency *in vitro*. Compound **2j** determined a G_2/M arrest of the cell cycle and also activated a strong apoptotic response. The β-tubulin immunofluorescence analysis indicated that compound **2j** effectively inhibited the microtubule organization in all cancer cell lines causing the formation of abnormal spindle, while did not affect the normal human fibroblast cells, NB1, Mrc-5 and IBR3. For all these reasons, compound **2j** could be a good candidate in chemopreventive or chemotherapeutic strategies.

INTRODUCTION

Among diseases, cancer is not a simple pathological state, but it can be identified as a broad group of diseases characterized by high proliferative index and the spreading of aberrant cell from their site of origin.[1] This malignant disease is considered the second major leading cause of death worldwide after cardiovascular desease,[2] to be noted that cancer mortality began decreasing in 1992.[3,4] Clinically, the therapeutic treatment of cancer is usually a combination of surgery and/or radiotherapy with chemotherapy.[5]

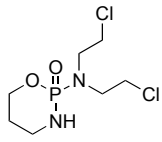
Current chemotherapy consists of drugs that are placed into three major periods, each with distinctive antitumor activity levels of action and toxicity.[6]

The first period (classical chemotherapeutics) contains drugs, which mainly blocked DNA replication and cell division, such as DNA cross-linking agents, mitosis inhibitors or topoisomerase poisons.

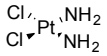
The second period is characterized by drugs, which directly interact with signaling intermediates contributing to cancer growth, most notably tyrosine kinase and related inhibitors.

In the third period the drugs are categorized to target broad cellular machineries with a mode of action that is not directly associated with DNA replication or cell division, but they are involved in the interaction with nucleosome, proteasome, protein chaperones or DNA repair process such as histone deacetylase inhibitors, DNA methyltransferase inhibitors, proteasome inhibitors or PARP inhibitors (Figure 1).

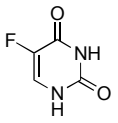
a) First period antitumor agents



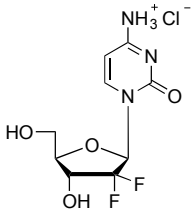
Cyclophosphamide[7]
(alkylating agents,
nitrogen mustard)



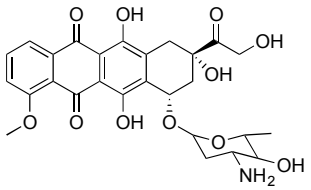
Cisplatin[7]
(organoplatinum complex)



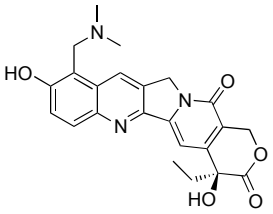
5-Fluorouracil[8]
(antimetabolites,
pyrimidine antagonist)



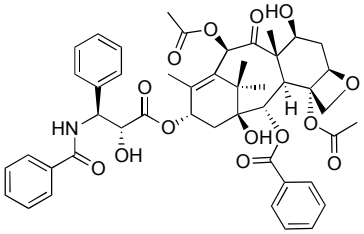
Gemcitabine[9]
(nucleoside analogues
DNA polymerase inhibitor)



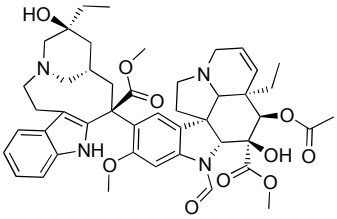
Doxorubicin[10]
(anthracyclines,
topoisomerase inhibitors)



Topotecan[11]
(camptothecins,
topoisomerase poison)

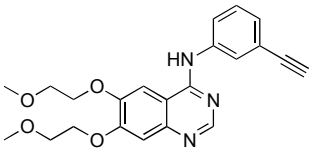


Paclitaxel[11]
(taxanes,
mitosis inhibitors)

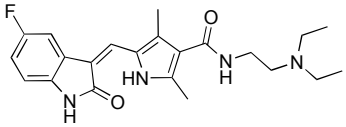


Vincristine[11]
(vinca alkaloids
mitosis inhibitor)

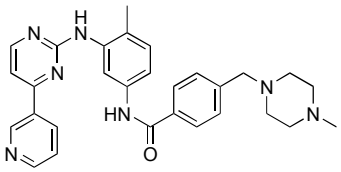
b) Second period antitumor agents



Erlotinib[12,13]
(EGFR inhibitor)

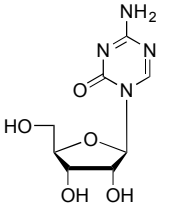


Sunitinib[12,13]
(VEGFR inhibitor)

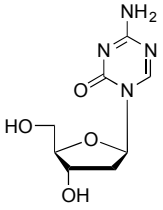


Imatinib[12,13]
(Bcr-Abl inhibitor)

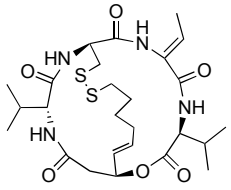
c) Third period antitumor agents



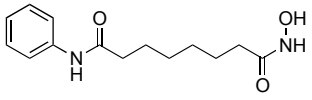
5-Azacytidine[14]
(Chromatin modification agent
DNA methyltrasferase inhibitor)



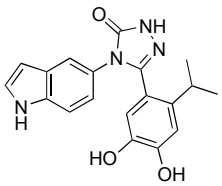
Decitabine[14]
(Chromatin modification agent
DNA methyltrasferase inhibitor)



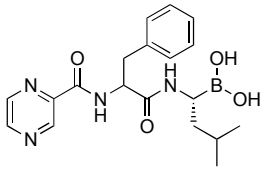
Romidepsin[14]
(Chromatin modification agent
HDAC inhibitor)



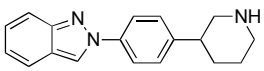
Vorinostat[14]
(Chromatin modification agent
HDAC inhibitor)



Ganetespib[15]
(Protein folding agent
HSP90 inhibitor)



Bortezomib[16]
(Proteasome function agent
Proteasome inhibitor)



Nirafarib[17]
(DNA repair agent
PARP inhibitor)

Figure 1. Selected drugs belonging to the three periods.

The therapeutic use of chemotherapy agents can lead to common complications arising from clinical systemic toxicity and development of resistance. Systemic toxicity associated with anticancer drugs is usually observed in the bone marrow, the gastro-intestinal (GI) tract and hair. Therefore, the search for novel chemical structures, with broader therapeutic windows and acceptable resistance profiles, are being actively pursued.

In a previous paper, we reported the preparation and the antiproliferative effect of a series of 2,5-disubstituted 1,3,4-oxadiazole.[17] This study indicated that these compounds, **1a,b** (Figure 2), with one nitrogen tricyclic heterocycle at position 2 and a phenyl moiety at position 5 exhibited considerable cytotoxicity *in vitro*.

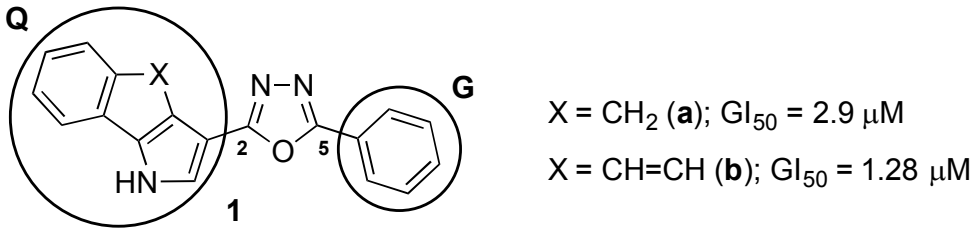
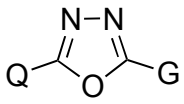


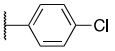
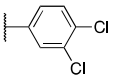
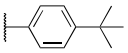
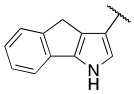
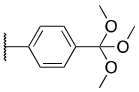
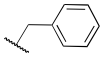
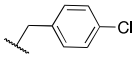
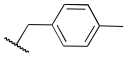
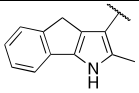
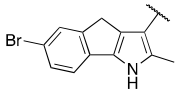
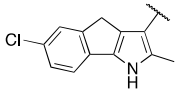
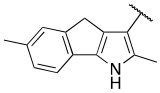
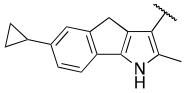
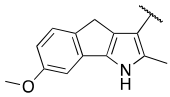
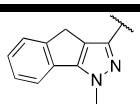
Figure 2. Compounds **1a,b**.

Although the mechanism of action of the oxadiazoles **1a,b** is probably multimodal in nature, our previous study indicated that an antimitotic activity with microtubule polymerization inhibition may be a major cellular event. In continuation of our research in this field, we wondered whether the introduction of different side-arm substitution patterns at the 2 and 5 positions of the 1,3,4-oxadiazole pharmacophore might provide compounds with interesting anticellular features.

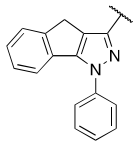
In the report, we described novel unsymmetrically disubstituted 1,3,4-oxadiazole of general structure **2** (see Table 1). The biological evaluations of the antitumor activities of the unsymmetrical analogues **2a-q** have been performed and the results will be discussed.

Table 1. 1,3,4-Oxadiazoles 2,5 disubstituted 2a-l



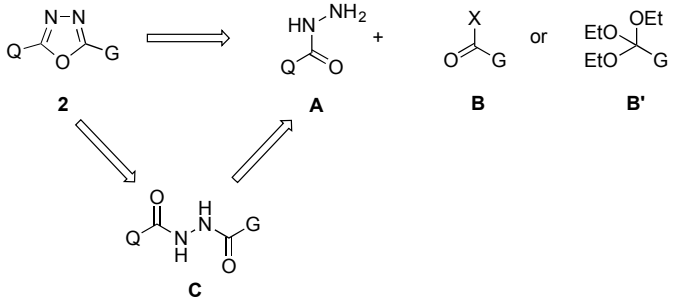
Compounds	Q	G
2a		
2b		
2c		
2d		
2e		
2f		
2g		
2h		
2i		
2j		
2k		
2l		
2m		
2n		
2o		
2p		

2q



CHEMISTRY

Scheme 1. Retrosynthesis of disubstituted oxadiazoles 2



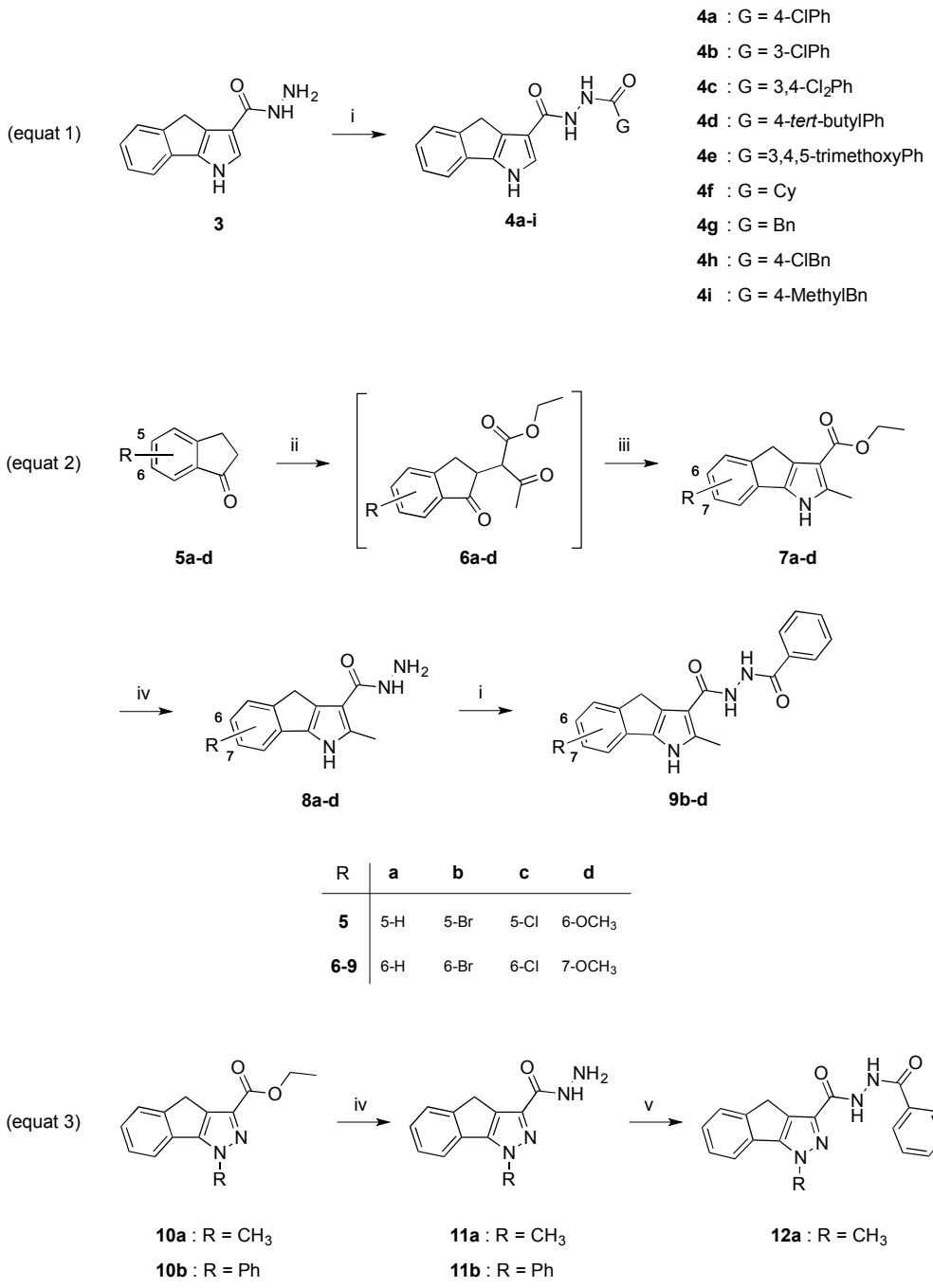
The oxadiazoles described in this report were prepared classically by the straightforward and operationally simple method shown in the retrosynthetic sense in Scheme 1. 1,3,4-Oxadiazoles **2** were synthesized by direct coupling of the appropriately substituted hydrazide **A** to the necessary acid derivative **B** or triethylorthoformiate **B'**. Alternatively, the hydrazide **A** were converted to the acyl hydrazide **C** with acid derivative **B** and then cyclized to provide 1,3,4-oxadiazoles **2**. The hydrazides **8a**, **11b** and acyl hydrazides **4a-i**, **9b-d** and **12a**, used in preparation of the 1,3,4-oxadiazoles in Table 1 were synthesized as illustrated in Scheme 2.

For example, treatment of hydrazide **3** in combination with an acid in the presence of appropriate coupling reagent such as an acid chloride and pyridine or EDCI and HOBt provided 1,4-dihydroindeno[1,2-*b*]pyrrole acyl hydrazides **4a-i** (Scheme 2, equat 1).

Equation 2 afforded the synthesis of intermediates **8a-d** and **9b-d**. These molecules were easily obtained starting from ketones **5a-d** that, by a click reaction, were converted into the corresponding tricyclic scaffolds passing through the acyl derivatives **6a-d**. **8a-d** were achieved with different time of reaction from **7a** (24 h) and **7b-d** (72 h) using hydrazine hydrate. **8a** was directly used for the next step (see scheme 3), whereas **8b-d** didn't react with ethyl orthobenzoate in the same manner of

8a so were transformed in the corresponding acyl hydrazides **9b-d**, as previously described in equation 1.

Scheme 2. Preparation of hydrazides and acylhydrazides[a]

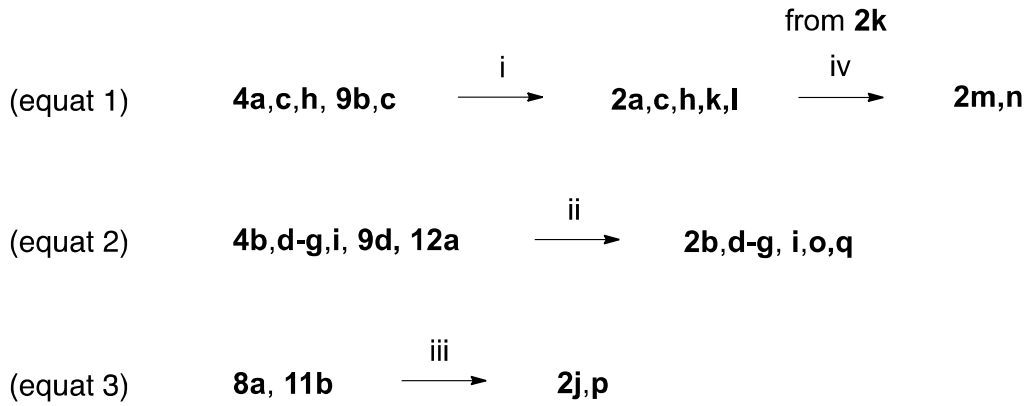


[a] Reagents and conditions: (i) ArCOCl, NMP, rt, 12-48 h (for **4a**, **4c**, **4f**, **4g**, **8b-d**) or ArCOCl, Py, DCM, rt, 24-30 h (for **4b**, **4d**, **4e**) or $ArCH_2COOH$, EDCI, HOBt, NMM, DMF/DCM, rt, 24 h for

(**4h**, **4i**); (ii) EAA, NaH, THF anhydrous, rt, overnight; (iii) $AcONH_4$, EtOH, 80 °C, 12 h; (iv) NH_2-$NH_2 \cdot H_2O$, dry EtOH, reflux, 24-72 h; (v) PhCOCl, Py, DCM, rt, 2 h.

Reaction of 1-methyl and 1-phenyl-1,4-dihydroindeno[1,2-*c*]pyrazole, **10a** and **10b**, (Scheme 2, equat 3) with hydrazine hydrate in ethanol furnished the desired hydrazides **11a**,**b**. **11a** on further reaction with benzoyl chloride yielded the required acylhydrazide **12a**.

Scheme 3. Preparation of 1,3,4-oxadiazoles 2a-q in Table 1



[a] Reagents and conditions: (i) $POCl_3$, 110 °C, 2 h; (ii) CH_3CN, TEA, CCl_4, PPh_3, rt, 6-48 h; (iii) $PhC(OEt)_3$, DMF, reflux, 3-24 h; (iv) **2k**, alkylboronic acid, $Pd(OAc)_2$, Py_3P, K_3PO_4, toluene, H_2O, MW, 130 °C, 5 h.

The 1,3,4-oxadiazoles in Table 1 were prepared according to the methods in Scheme 3. 1,3,4-oxadiazoles **2a**, **2c**, **2h**, **2k** and **2l** (Scheme 3, equat 1) were prepared by heating acyl hydrazides **4a**,**c**,**h** and **9b,c** with phosphorous oxychloride. Compounds **2m,n** were obtained from **2k** by a Suzuki reaction in presence methylboronic acid (for **2m**) or cyclopropylboronic acid (for **2n)**, tricyclohexylphosphine and $Pd(OAc)_2$.

Cyclization of compounds **4b**,**d-g**,**i, 9d** and **12a** (Scheme 3, equat 2) was performed using triphenylphosphine and triethylamine in dichloromethane affording 1,3,4-oxadiazoles **2b**,**d-g**,**i,o**

and **2q**. The desired 1,3,4-oxadiazoles **2j** and **2p** (Scheme 3, equat 3) were obtained directly from hydrazides **8a** and **11b** by reaction with triethyl orthobenzoate in refluxing dimethylformamide.

BIOLOGY

Antiproliferative activity against different human cancer cell lines.

Preliminary screening of the synthesized compounds (**2a-q**) was performed to evaluate cytotoxic activity against selected human cancer cell lines, Mfc7 (breast cancer) and HeLa (cervix adenocarcinoma) by employing a colorimetric assay (XTT) for the quantification of cell proliferation and viability. The cells were incubated for 72 hours with 10-5-2 μM of individual compounds, or doxorubicin (referenced drug), or DMSO (molecules' solvent, used as control). The results of this cytotoxicity data at three different doses in comparison with positive controls, such as **1a** and doxorubicin, are reported in Figure 3.

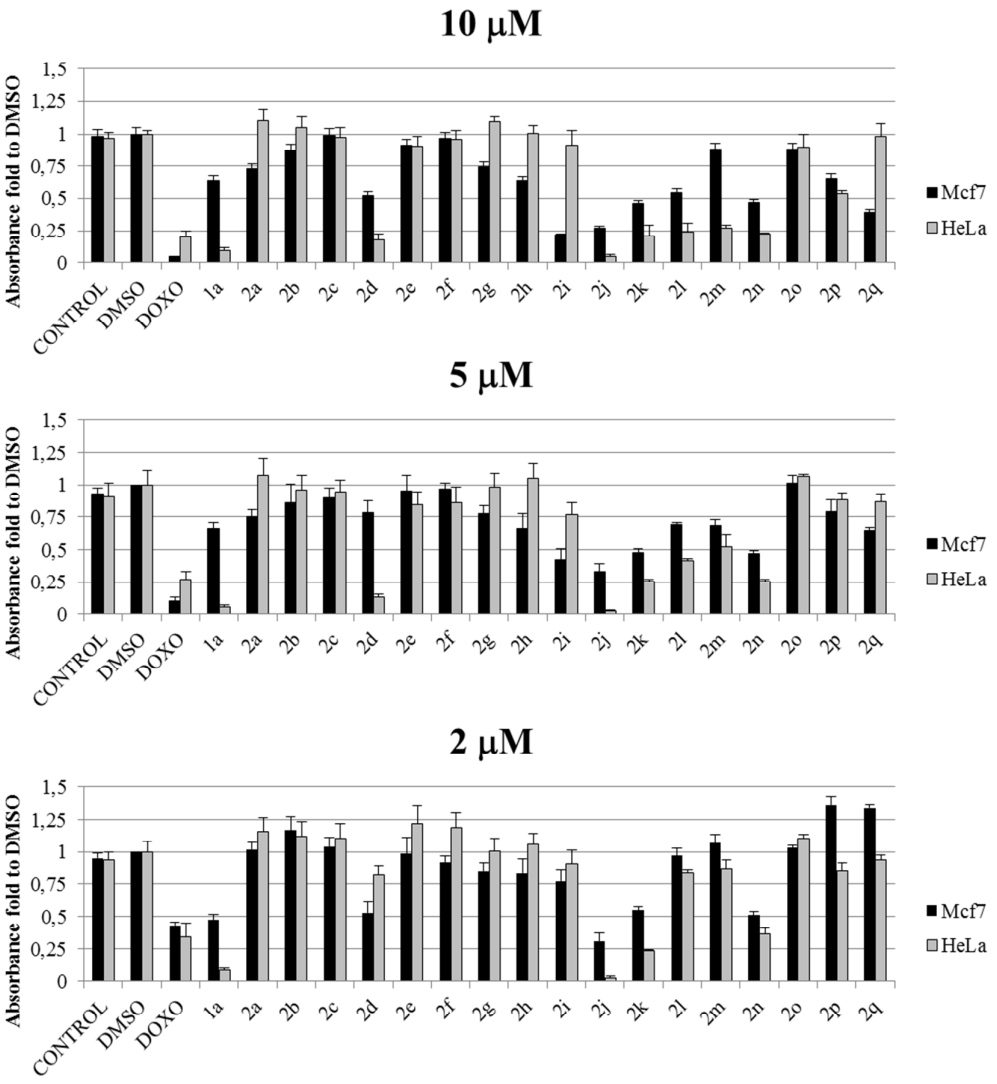


Figure 3. Inhibition of tumor cell proliferation *in vitro* as determined by XTT assay. All the compounds were dissolved in dimethylsulphoxide (DMSO). Mcf7 and HeLa were incubated with DMSO (vehicle control), or different concentrations (10-5-2 μM) of individual compounds: **1a**, or distinctive compound **2a** to **2q**, or doxorubicin. Cells were seeded in 96 well plates at a density of $1,5/2\times10^3$ cells/well. After 24 hours of cultivation, the adhered cells were treated with the compounds and incubated for 72 hours. The percentages of viable cell were calculated by comparing treated and control cells. Cell viability is represented in relation to DMSO and XTT assays were repeated 3 times.

To evaluate the effect of the bioisosteric replacement of the pyrazole nucleous of **1a**, first we synthesized compound **2a** and analogues **2b-i**. Among the nine compounds **2a-i**, derivative **2d**, bearing a 1,4-dihydroindenopyrrole scaffold at C_2 of the central oxadiazole core together with a phenyl side chain substituent attached to the C_5, showed significant activity against the two tumor cell lines (IC_{50} = 3.4 μM for HeLa and IC_{50} = 9.5 μM for Mcf7) (Figure 3).

To further evaluate the effect of substitution on the 1,4-dihydroindenopyrazole system of **1a**, derivatives **2j-o** were synthetized. Interestingly, only the 2'-methyldihydroindenopyrrole compound **2j,** at 2 μM, had better activity in both tumor cells (IC_{50} = 0.05 μM for HeLa and IC_{50} = 1.7 μM for Mcf7) showing higher antiproliferative activity than **1a** and reference drug doxorubicin.

Their calculated IC_{50} values are listed in Table 2. The bromine substitution at the C_6-position (**2k**) gave comparable potency (IC_{50} = 0.51 μM for HeLa and IC_{50} = 4.26 μM for Mcf7) to compound **2j**, whereas the less lipophilic chlorine derivative **2l** appeared less favorable (IC_{50} = 45 μM for HeLa and IC_{50} >10 μM for Mcf7). Replacement of the bromine with less and more lipophilic alkyl groups such as methyl (**2m**) or cyclopropyl (**2n**) moieties provided the cyclopropyl analogue **2n** that was equipotent (IC_{50} = 1.58 μM for HeLa and IC_{50} = 2.31 μM for Mcf7) respect to **2j**. Introduction of a hydrophilic methoxy group at the C_7-position of the tricyclic ring system lowered the potency. Among these compounds the degree of anticellular potency increases in the sequence OCH_3<Cl<CH_3<Br<cyclopropyl<H.

The above findings suggest that lipophilic substituents on the benzyl ring of tricyclic moiety of **2j** may favorably influence *in vitro* activity.

We also briefly explored the effect that tricyclic ring system replacement had on cytotoxic activity. Compounds **2p,q** had a 1,4-dihydroindenopyrazole moiety as **1a**, but they displayed a minor to significant decrease in cell viability when compared both to **1a** and to the 2-methyl-1,4-dihydroindenopyrrole analogue **2j (**Figure 3).

As a result, an interesting and large impact on cytotoxic activity was found for 2 and 5 substitution of the central 1,3,4-oxadiazole core. Whereas 5-substitution with different groups as 4-Cl-phenyl, 4-*tert*butylphenyl, trimethoxyphenyl, cycloexyl or benzyl had minimal effects on cytotoxic activity, 2-substitution of dihydroindenopyrrole system with a dihydroindenopyrazole moiety markedly reduced the cytotoxic activity. Thus, compound **2j** exhibited the highest anticellular activity among all the new compounds tested in this study.

Table 2. Cytotoxic activities of 1a and 2a-q[a]

Compound	IC_{50} Mcf7	IC_{50} HeLa
1a	2.1 μM	0.5 μM
2a	> 10 μM	> 10 μM
2b	> 10 μM	> 10 μM
2c	> 10 μM	> 10 μM
2d	9.5 μM	3.4 μM
2e	> 10 μM	> 10 μM
2f	> 10 μM	> 10 μM
2g	> 10 μM	> 10 μM
2h	> 10 μM	> 10 μM
2i	4.25 μM	> 10 μM
2j	1.7 μM	0.05 μM
2k	4.26 μM	0.51 μM
2l	> 10 μM	4.5 μM
2m	> 10 μM	5.3 μM
2n	2,31 μM	1.58 μM
2o	> 10 μM	> 10 μM
2p	> 10 μM	> 10 μM
2q	7.59 μM	> 10 μM

[a] Cytotoxic activities of the new compounds as given by IC_{50} values in selected cancer cell lines HeLa and Mcf7.

Compound **2j** was found to be the most interesting and potent of the series; thus, its IC_{50} value was determined and its cytotoxicity evaluated by comparing its activities in a panel of 11 different cancer cell lines as summarized in Table 3. Compound **2j** was considered highly cytotoxic, with IC_{50} values of < 1.7 μM. The observed activity pattern was quite variable among different cancer cell lines.

Table 3. Cytotoxic activities of 1a and 2j[a]

	Cell line	IC_{50} **1a**	IC_{50} **2j**
HeLa	Cervix Adenocarcinoma	0.5 ± 0.02 μM	0.05 ± 0.03 μM
RD	Rhabdomyosarcoma	0.4 ± 0.05 μM	0.06 ± 0.04 μM
T98G	Gliobastoma multiforme	0.5 ± 0.03 μM	0.06 ± 0.02 μM
SH-SY5Y	Neuroblastoma	0.69 ± 0.06 μM	0.07 ± 0.04 μM
H1299	Non small lung cancer	0.9 ± 0.04 μM	0.12 ± 0.05 μM
SKNAS	Neuroblastoma	0.76 ± 0.04 μM	0.19 ± 0.06 μM
A549	Non small lung cancer	1 ± 0.07 μM	0.24 ± 0.08 μM
Kelly	Neuroblastoma	1.5 ± 0.09 μM	0.25 ± 0.04 μM
LAN1	Neuroblastoma	0.78 ± 0.05 μM	0.38 ± 0.07 μM
PC3	Prostate cancer	1.9 ± 0.08 μM	0.67 ± 0.04 μM
Mcf7	Breast cancer	2.1 ± 0.12 μM	1.7 ± 0.09 μM

[a] Cytotoxic activities of **2j** as given by IC_{50} values in selected cell lines of a panel of 11 different cancers. Molecule **2j** inhibited tumor cell proliferation in all cell lines with IC_{50} values between 0.05 and 1.7 μM.

Compound **2j** was selective as it was 28-34 times more active against HeLa, RD and T98G cell than against Mcf7 cells. The activity of **2j** against H1299, SH-SY5Y and SKNAS cells was high with IC_{50} ranging from 0.12 to 0.19 μM. Moreover, also A549, Kelly, Lan-1 and PC3 cells were sensitive to **2j** with IC_{50} values of 0.24, 0.25, 0.38 and 0.67 μM, respectively.

Since nonspecific site of action and toxicity of drugs represent two of the main problems in chemotherapy, comparable studies were carried on human dermal and lung fibroblast cells (NB1, Mrc-5, IBR3) used as control, and on the corresponding immortalized cell lines (NB1 h-TERT, Mrc5 SV1 S40, IBR3-G S40).

As shown in Table 4, it is interesting to note that active derivative **2j** exhibited selectively cytotoxicity to cancer cells, with a weak effect on human fibroblast cells, used as control. Therefore in all normal analyzed cell lines, compared to the corresponding immortalized non-tumorigenic cells, compound **2j** revealed less sensitivity and a higher IC_{50}.

Table 4. Cytotoxic activities of 1a and 2j

	Cell line	IC_{50} **1a**	IC_{50} **2j**
NB1	Primary human dermal	1.8 ± 0.08 μM	1.3 ± 0.06 μM
NB1 h-TERT	Immortalized human	1.6 ± 0.05 μM	0.13 ± 0.03 μM
IBR3-G	Primary human dermal	1.6 ± 0.07 μM	0.77 ± 0.06 μM
IBR3-G SV40	Immortalized human	1.8 ± 0.08 μM	0.21 ± 0.04 μM
Mrc5	Primary human lung	1.9 ± 0.05 μM	0.19 ± 0.05 μM
Mrc5-SV1 S40	Immortalized human	1.7 ± 0.07 μM	0.13 ± 0.03 μM

Table 4: The IC_{50} values for the synthesized compounds **1a** and **2j** against human normal and immortalized cell lines show that primary cells are less sensitive than the same immortalized cell lines.

Compound 2j inhibit colony formation in cancer cells.

The cellular long-term effects of **1a** and **2j** were tested in all the cell lines by determining their ability to inhibit colony formations after drug washout. This experimental paradigm displays a cancer cell ability to produce a viable colony after treatment and it is a mean that may help to predict efficacy *in vivo*.[19,20]

The cellular persistence of **1a** and **2j** and the number of colonies formed after two weeks of exposition in a medium with the drugs were determined using a clonogenic assay. Clonal growth of all cell lines was inhibited by **2j** in a dose dependent manner. Representative pictures of colonies

following treatment with 0.3 μM **1a** or 0.03 μM **2j** are shown in Figure 4. At low concentration, like 0.03 μM, only compound **2j** was able to potently inhibit up to 50% of colony formation inducing cytotoxicity and suggesting a high level of cellular persistence. Conversely, **1a** was not able to inhibit clonogenic growth under the same conditions, but only at the highest concentration. These results are consistent with the data obtained in the *in vitro* antiproliferative activity and the IC_{50} values of compounds **1a** and **2j**. The data also confirm the greater potency and activity of compound **2j** compared to **1a.**

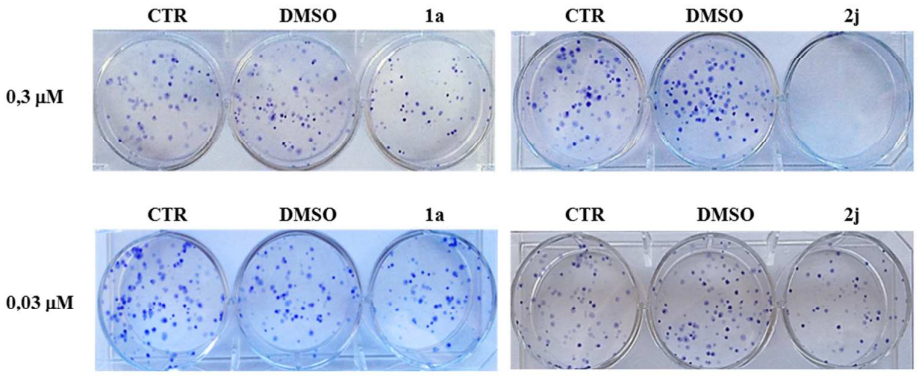


Figure 4. Representative photographs from one of the triplicate demonstrating the effects of exposure to **1a** and **2j** on colony formation. HeLa were treated with DMSO, **1a** and **2j** at 0.3 and 0.03 μM. Colonies have been fixed and stained with glutaraldehyde and crystal violet, two weeks after cell treatment.

Inhibitor agents of ABC transporters improve 2j effects on resistance cell lines

The phenomenon of multidrug resistance (MDR) in the treatment of malignant tumors is a distinguished problem in oncology whereby cancers become resistant to various agents, consequently requires careful consideration in cancer therapy.[21]

Tumor cells adopt several mechanisms to evade death induced by anti-tumor agents. These include changes in apoptotic pathways and activation of cell-cycle checkpoints to increase DNA repair.

Alternatively, cancer cells develop resistance by the up-regulation of a family of transmembrane ATP binding cassette (ABC) transporter proteins that are present in all living organisms[22] and altered anticancer drug transport mechanisms. These specific proteins are able to trigger chemotherapy resistance carrying out in an active manner the extrusion of a large collection of therapeutic compounds from cancer cells. They bind ATP in their ATP binding domain and use the energy to transport various molecules across the cell. The ABC transporter family has been divided into seven subfamilies based on the sequence similarity as well as structural organization.[23] Among these proteins, P-glycoprotein (Pgp, ABCB1), multidrug resistance-associated protein (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2) are chiefly responsible for drug resistance in tumor cells.

In literature it is just known that the beta-carboline alkaloid Harmine reduced resistance to some anticancer drugs mediated by BCRP and might be an interesting new reversal agent.[24] Furthermore, Reversan inhibits MRP1 and P-glycoprotein (Pgp) very electively.[25, 26]

In this study, we show that the addition of small molecule inhibitors of MRP1 and BCRP had a significant effect on cancer cell response to **2j**.

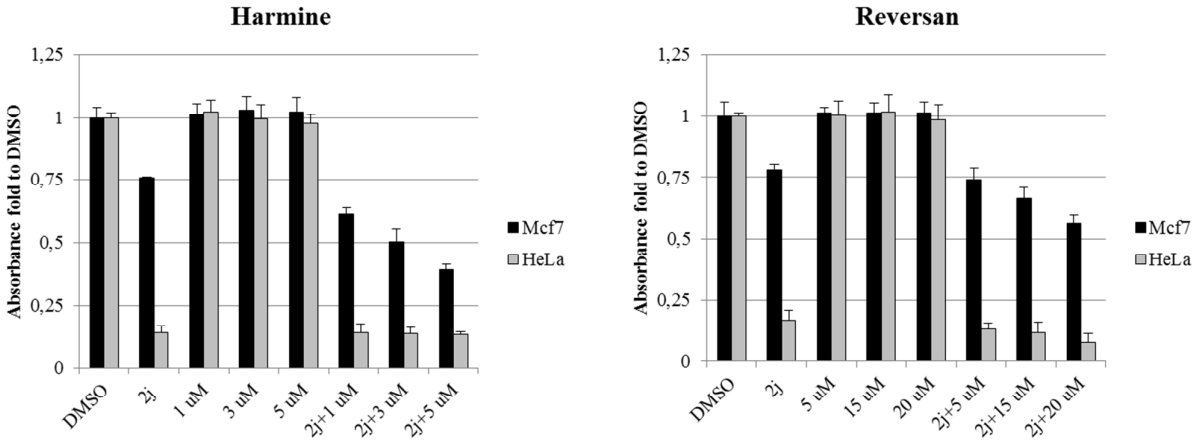


Figure 5. Effects of the inhibitors of MRP1 and BCRP on HeLa and Mcf7. Cell lines were treated with **2j** (0.5 □M), Harmine (1-3-5 □M) or Reversan (5-15-25 □M) or co-treated with **2j** + Harmine or **2j** + Reversan for 72 hours, and tested for chemosensitivity using XTT assay**.**

As illustrated in Figure 5, we treated the cell lines with different non-toxic concentrations of Harmine or Reversan as previously suggested.[24,27] Considering the different **2j** IC_{50} values for HeLa and Mcf7, cell lines were also treated with 0.5 □M of **2j**, a concentration that allowed a–clear inhibitory effect in both cell lines.

Evaluation of XTT assay indicated that in Mcf7, the cells more resistance to **2j**, the co-treatment of Harmine or Reversan with **2j**, significantly inhibited cell proliferation with higher activity than **2j** alone. Specifically, the most notably enhanced cell death was evident when Mcf7 cells were treated with a combination of **2j** and Harmine (5 □M). This co-treatment was able to potently inhibit until 40% of proliferation improving response to cell death comparing than **2j** alone.

However, in HeLa, the most sensitive cells to **2j** treatment, inhibition of ABC transporters by Reversan and Harmine did not lead to a significant change in drug-induced cell death on **2j** drug response.

These results possibly suggested that increased MRP1 and BCRP expression in some cell lines, such as Mcf7, contributes to chemoresistance through increased drug efflux and reduced bioavailability of the derivative **2j** within the cancerous cells. Improving intracellular exposure to efficient chemotherapeutics, through Harmine and Reversan, would significantly increase Mcf7 cell death when used in combination with compound **2j**.

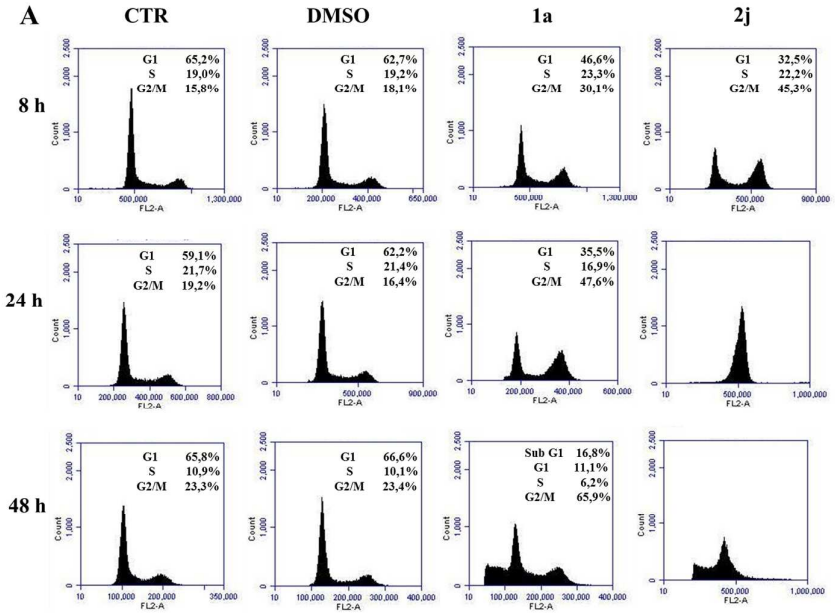
2j induces G_2/M phase cell cycle arrest

In order to investigate the cellular mechanisms of prototype **1a** and compound **2j**, a cell cycle distribution of all cell lines was analyzed by flow cytometry. From FACS analysis, a significant increase in G_2/M phase was observed in comparison to untreated control after **2j** treatment. Compound **2j** showed various effects on the different cell lines. After 2 hours treatment, G_2/M phase cell cycle arrest was observed in HeLa, RD and T98G, followed by gradual increase during the time point. This effect changed after 24 hours of treatment and the rapid arrest of cell growth

was followed by cell death. Also **1a** led to a G_2/M arrest but with less activity than **2j** (Figure 6A).

In Mcf7, PC3 and SH-SY5Y, **2j** induced a remarkable increase in G_2/M phase (from 24.2% to 78.1%) and a dramatic decrease in G_1 phase (from 54.1% to 15.7%) and S phase (from 21.7% to 6.3%) after 24 hours treatment. Interestingly, the G_2/M arrest persisted almost unchanged until 144 hours (Figure 6B).

In further cell lines, such as H1299, A549 and Lan-1 cell lines in comparison with the DMSO control group, 24 hours treatment of **2j** led to G_2/M arrest with 74.6% of cell lines in this phase compared with 22.2% in DMSO; 48 hours of treatment induced a decrease in G_2/M (42.9% with **2j** in comparison to 11.8% DMSO), a reduction in G_1 phase (34.3% with **2j** compared to 81% with DMSO) and an increase (13.4% of cell population) in sub-G_1 (Figure 6C).



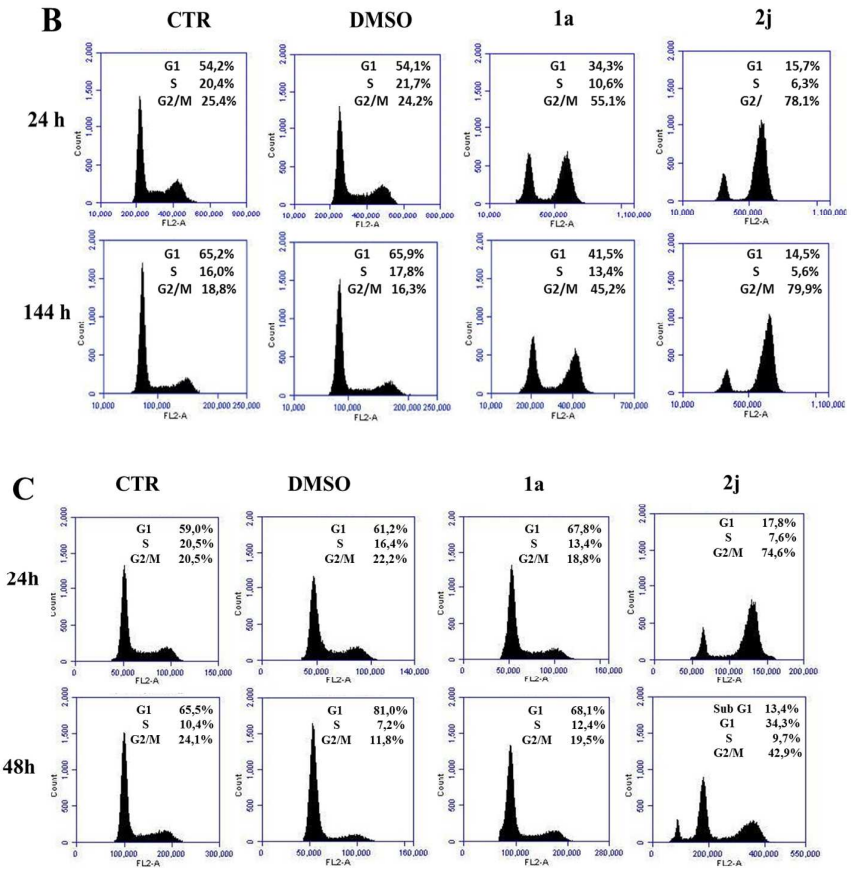


Figure 6. Cell cycle distribution of HeLa (A), Mcf7 (B) and H1299 (C) cancer cell lines after treatment with prototype **1a** and **2j**. Profiles were obtained by flow cytometry analysis using the DNA intercalating dye, propidium iodide (PI) to monitor cell cycle progression on the basis of DNA quantitation.

As shown in Table 5, the results obtained with the FACS analysis specify that the promising anticancer activity of **2j** may be ascribed to the G_2/M arrest.

Table 5. Distribution of cells in every phases of cell cycle after 1a and 2j treatment.

Cell line	Compound	Sub G_1	G_1	S	G_2/M
HeLa	CTR		59.1±2.12	21.7±1.67	19.2±1.09
	DMSO		62.2±1.34	21.4±1.94	16.4±2.15
	1a		35.5±1.63	16.9±0.96	47.6±1.28
	2j				
RD	CTR		55.1±1.93	15.9±2.18	29.0±1.37
	DMSO		56.7±1.48	14.4±0.92	28.9±1.93

	1a	2.4±0.93	42.9±1.79	18.6±1.05	36.1±1.57
	2j	6.1±0.58	36.9±2.06	10.6±0.89	46.4±1.28
T98G	CTR		83.0±1.53	6.1±0.79	10.9±1.20
	DMSO		86.1±1.29	5.1±0.82	8.8±0.93
	1a	3.3±0.63	38.7±1.69	9.6±1.03	48.4±0.79
	2j	16.8±0.94	11.1±1.37	6.2±0.88	65.9±0.69
H1299	CTR		59.0±1.95	20.5±1.34	20.5±1.14
	DMSO		61.2±1.69	16.4±1.76	22.2±1.74
	1a		67.8±1.49	13.4±1.37	18.8±0.93
	2j		17.8±1.42	7.6±1.57	74.6±0.64
SH-SY5Y	CTR		62.8±2.04	13.7±1.03	23.5±1.32
	DMSO		59.6±1.94	14.3±1.52	26.2±1.27
	1a		81.3±1.37	3.0±0.77	15.7±1.05
	2j	15.7±0.91	24.8±1.04	15.6±1.01	43.9±1.36
SKNAS	CTR		52.5±1.66	22.1±0.99	25.4±1.13
	DMSO		51.5±1.39	21.3±1.14	25.2±1.32
	1a		22.1±1.02	26.5±1.32	51.4±1.28
	2j	6.3±0.73	10.8±0.63	9.9±0.84	73.0±2.06
A549	CTR		64.8±2.03	11.7±1.14	23.5±1.26
	DMSO		65.4±1.84	7.6±0.63	27.0±1.32
	1a		70.1±2.16	9.4±0.77	20.5±1.18
	2j	13.8±0.86	11.4±0.93	9.5±0.82	65.3±1.66
Kelly	CTR		62.7±1.63	17.5±1.34	19.8±1.36
	DMSO		65.9±1.87	16.1±1.28	18.0±1.28
	1a		54.7±1.49	3.8±0.74	31.5±1.74
	2j	13.4±0.94	34.3±1.42	9.7±0.91	42.9±1.19
Lan-1	CTR		49.8±1.15	22.8±1.12	27.4±1.21
	DMSO		60.3±1.82	23.5±1.32	26.2±1.92
	1a		33.6±1.64	29.7±1.15	34.7±1.64
	2j		11.8±1.06	5.6±0.95	82.6±2.27
PC3	CTR		66.0±1.85	4.8±0.73	29.2±1.73
	DMSO		67.0±1.47	6.2±0.94	26.8±1.69
	1a		58.6±1.38	8.1±1.02	33.3±1.45
	2j		23.3±1.19	4.7±1.07	72.0±1.99
Mcf7	CTR		54.2±1.60	20.4±1.28	25.4±1.48
	DMSO		54.1±1.52	21.7±1.42	24.2±1.62
	1a		34.3±1.29	10.6±1.69	55.1±1.73
	2j		15.7±1.17	6.3±0.95	78.1±2.16

Table 5. FACS analysis of untreated and treated (DMSO, **1a**, **2j**) cancer cell lines showing the distribution of cells in various phases of cell cycle. The table shows the percentage of cells in each phase after 24 hours exposure to the compound at a concentration 1 μM.

Moreover, in order to evaluate whether **2j** effect is reversible or irreversible, cells were exposed to **2j** for 24 hours followed by replacement with normal cultured media (Figure 6A). The results

shown in Figure 6B, suggested that the G_2/M arrest caused by **2j** is reversible and its effect is cytostatic and not cytotoxic. After being changed with fresh medium, the cell cycle showed a comparable distribution among normal and control cycle (G_2/M phase: 59.5% after 24 hours **2j** treatment and 31.7% after 24 hours treatment + 24 hours change medium; 28.1% after DMSO treatment and 26.4% with DMSO treatment + 24 hours change medium).

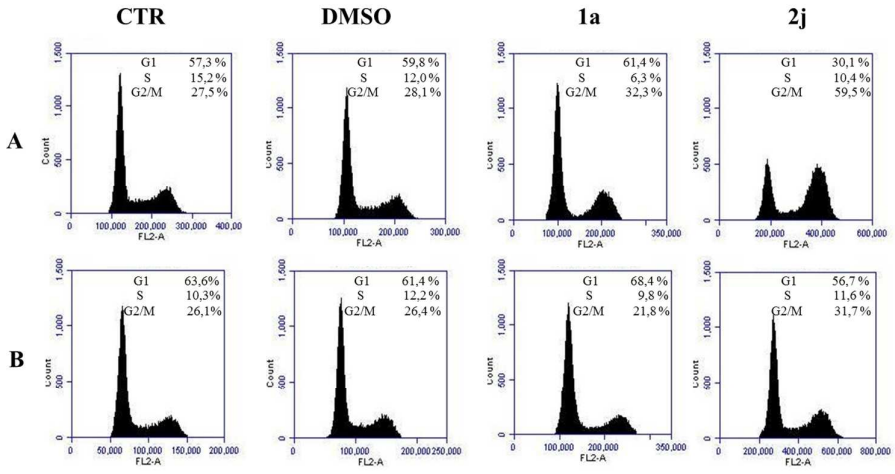


Figure 7. Effects of **1a** and **2j** on cell cycle distribution of Mcf7 cells after 24 hours of treatment with vehicle, **1a** or **2j** (A), and after 24 hours of treatment with compounds, followed by 24 hours of treatment with normal medium (B).

Furthermore, it is interesting that **2j** does not appear to be involved in G_2/M arrest on primary cell lines as shown in Figure 8A. Cells treated with **2j** from 24 to 72 hours were not significantly influenced by the treatment and the distribution of population among all the phases of cell cycle appeared similar to the control and DMSO (Figure 8A). When immortalized cells were treated with **2j** under the same conditions, a reduction of the percentages of G_1 and G_2/M arrested cells was observed. Interestingly, 25% of immortalized cells displayed a sub-G_1 peak after 48 and 72 hours exposure to the treatment of **2j** (Figure 8B).

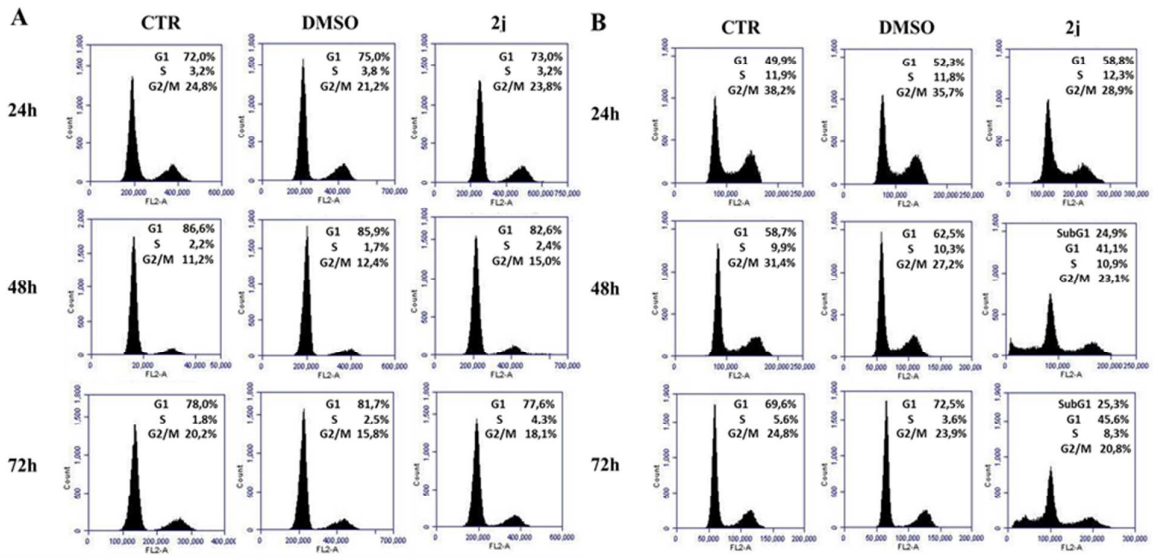


Figure 8. Flow cytometric analysis in primary NB1 (A) and immortalized NB1 h-TERT (B) cell lines after treatment with **2j** on cell cycle.

Compound 2j induce alteration on tubulin organization

Suggested by the evident G_2/M cell cycle arrest, the effect of **1a** and **2j** in a mitotic spindle microtubules, and overall cellular morphology were evaluated by immunofluorescence analyses using β-tubulin antibody.

Cells were treated with the compounds for 24 hours at the concentration of 1 μM, subsequently were permeabilized, fixed, and stained with β-tubulin and DAPI for DNA stain.

Deregulation of tubulin network after **2j** treatment was observed whereas no particular changes in β-tubulin organization were highlighted in both DMSO and in the control.

Different phenotypes were observed in cancer cell lines: Mcf7 showed an aberrant dissemination of β-tubulin, distinguished by uneven distribution around the nuclei, induction of micronucleated cells by **2j**, smaller and more compact nuclei as compared to the control.

In HeLa cell, low concentrations of **2j** (1 μM for **1a** and 0.1 μM for **2j**) induced abnormal cell division resulting in the formation of new daughter cells with tubulin structure but without nuclei or with incorrect β-tubulin organization and two nuclei.

Finally, SH-SY5Y revealed an irregular microtubule network with an atypical β-tubulin distribution. Figure 9 depicts the loss of its elongated structure with shorter microtubule bundles distributed around the nuclei and with apoptotic and pyknotic nuclei.

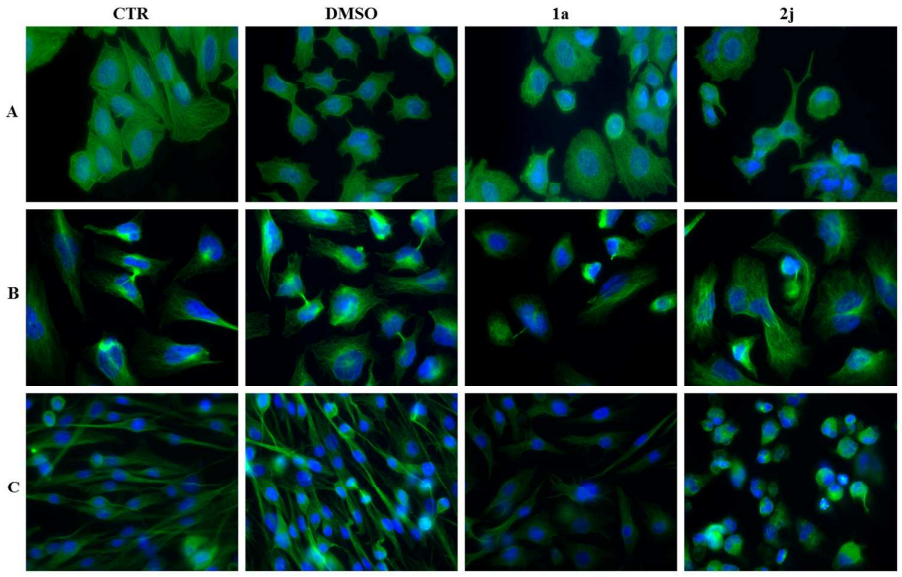


Figure 9. Representative images of immunofluorescence analysis showing β-tubulin expression (green) and DNA (blue) in various cancer cells Mcf7 (A), HeLa (B) and SH-SY5Y (C) after incubation with DMSO, **1a** and **2j**.

Molecule 2j increase p53 protein level in cancer cell lines.

In order to determine whether the anti-proliferative effects and the mechanisms associated with cell cycle arrest suggesting the involvement of p53, cells were treated with 1 μM of **2j** for 24 hours, lysed, and p53 protein levels were evaluated by immunoblotting and quantified by densitometric analysis using GAPDH as control. As shown in Figure 10, **2j** treatment enhances a substantial increase of p53 expression levels in all cancer cell lines, respectively 2 or 3 times higher compared to the untreated controls. Interestingly, no modulation in p53 expression was observed in HeLa because these cells contain no detectable p53 protein. Moreover, the data showed unaltered p53

levels for fibroblasts and a p53 deregulation, even low, for immortalized cells, confirming that **2j** preferentially inhibits the growth of cancer cells

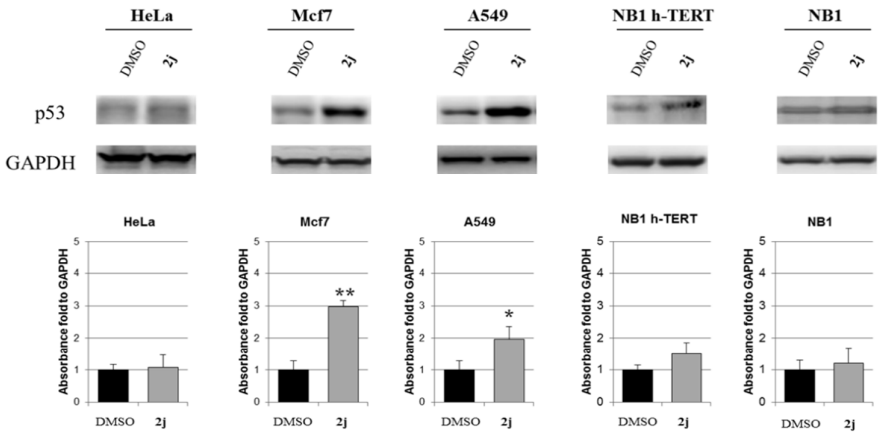


Figure 10. Overexpression of p53 protein in cancer (HeLa, Mcf7, A549), primary (NB1) and immortalized (NB1 h-TERT) cells after 24 hours of **2j** treatment. GAPDH is shown as a control of protein loading. The overexpression of p53 has been measured and the data displayed in a histogram. Columns are means of three different experiments.

Molecule 2j induced an apoptotic response in cancer cell lines.

To determine whether **2j** induces apoptosis, Annexin V-FITC/PI assay was performed. After treatment with 5 μM of **2j** for 48 or 72 hours, cells were double stained with Annexin V and PI and assayed by flow cytometry.

In Figure 11, the data show that compound **2j** induced apoptosis of cancer cells: in HeLa cells, after 48 hours, the rate of apoptosis in the control was 3.6% and in DMSO was 6.5%, while the percentage of apoptotic cells was 89.7% after treatment with 5 μM of **2j**.

In other cell lines such as A549, **2j** needs 72 hours of treatment to induce apoptosis with percentage of 52.7% respect to 0.9% in the control and in 6.5% in DMSO.

Interestingly, at the same doses and after 72 hours, only 6% of fibroblast and 11.8% of immortalized cells showed apoptotic effect respect to DMSO.

These results suggest that **2j** induced apoptosis and had a more potent effect in cancer cell lines; on the contrary, primary cells displayed a low sensitivity to the compound.

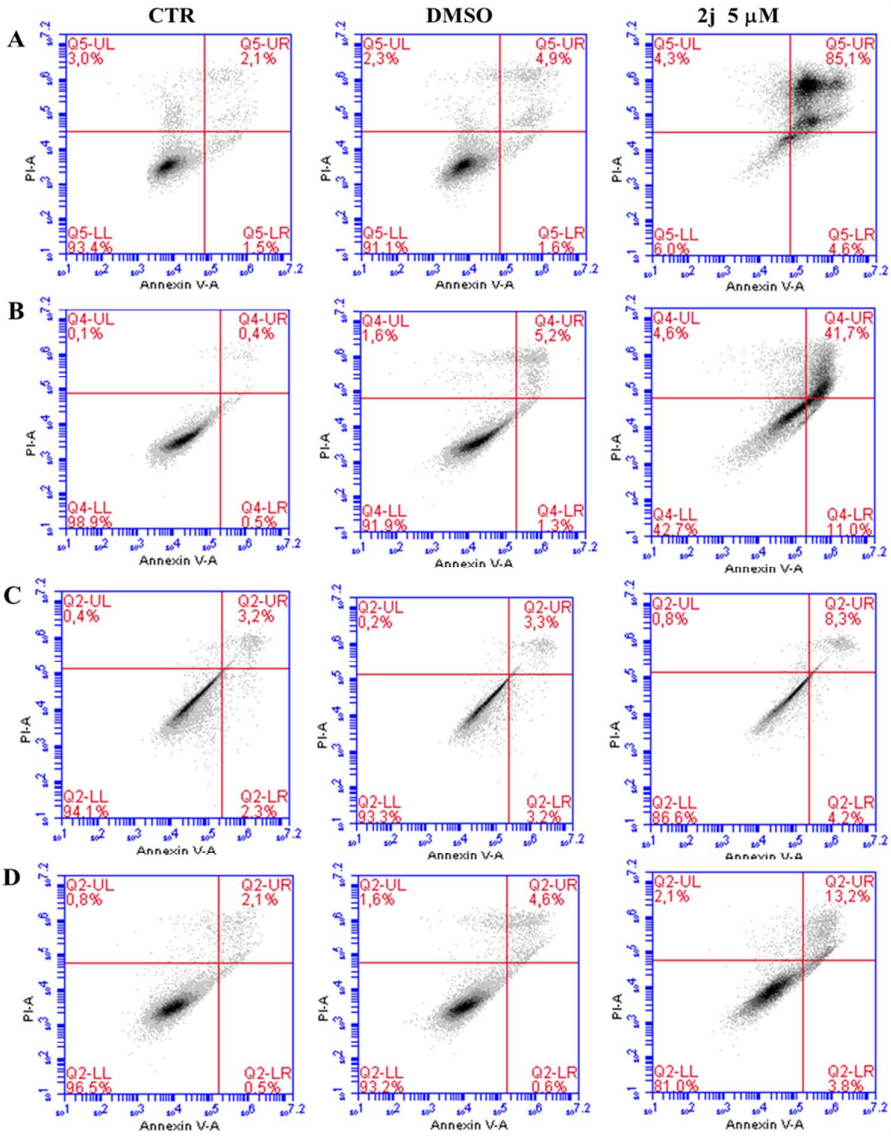


Figure 11. HeLa (A), A549 (B), NB1 (C), NB1 h-TERT (D) were cultured with **2j** at 5 □M for 48 (HeLa) or 72 hours. Cells were stained by Annexin V FITC/PI and apoptosis was analyzed by flow cytometry. The lower left quadrants show the vital cells; in the upper right quadrants the late apoptotic cells are indicated; the lower right quadrants show the early apoptotic cells and the upper left quadrants display the dead and necrotic cells.

DISCUSSION AND CONCLUSIONS

Seventeen new unsymmetrical 2,5-disubstituted 1,3,4-oxadiazoles were synthesized and screened as new potential anticancer candidates.

The antiproliferative effects of substituted **1a** derivatives were studied and it was found that compound 2-(2-methyl-1,4-dihydroindeno[1,2-*b*]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole, **2j**, exhibited the strongest anticancer activity against a panel of different human cancer cell lines with IC_{50} ranging from 0.05 to 1.7 μM, more effectively than **1a** and doxorubicin in inhibiting cancer cell growth.

The different potency of compound **2j** against several cell lines studied could be due to the ABC transporters, in fact they can increase the efflux of **2j** from cancer cells, decreasing the intracellular drug concentration. Modulators of ABC transporters, like Harmine and Reversan, have the potential to increase the efficacy of **2j** in the resistance cell lines.

Together, our data suggest that the designed molecule **2j** could be a prospective anticancer drug, thus, clearly display its ability to induce a solid antiproliferative effect, especially targeting cancer cells. Moreover our results demonstrate that all the fibroblasts were less sensitive to **2j** when treated at the same concentrations compared to immortalized cell lines, suggesting that this compound shows low systemic toxicity and preferential cytotoxic activity on cancer cells.

Usually, cells, grown in monolayer, are more sensitive to cytotoxic agents than cells grown in colonies due to the greater surface available to the chemotherapy agents, compared to the limited drug penetration in the colonies.[28] Interestingly, colony formation assay indicate that **2j** can abolish proliferation of cancer cells *in vitro,* in both monolayer and colony cell culture conditions. Moreover, our data suggest that **2j** could perhaps show anticancer activity *in vivo,* without affecting the proliferation of normal cells, a common limit of many anticancer drugs.[29]

Since deregulations of cell cycle progression are typical of cancer, many of the new drugs used as anticancer agents work at multiple steps in the cell cycle and their effects may be cytostatic or cytotoxic, depending on the cell cycle status of the target cells.[30,31] The knowledge of the molecular

interactions involved may suggest ways to alert cells to the effects of these compounds, hence the molecular and cellular target mechanisms utilized by **2j** were explored through flow cytometry analysis and immunofluorescence. FACS analysis highlighted a considerable G_2/M phase arrest, indicating an important role of **2j** during cell cycle distribution. On the contrary, normal fibroblasts treated with **2j** under the same conditions, did not show significant effects in terms of blocking the cell cycle, again notifying the favorite activity of **2j** on cancer cells.

Various studies have highlighted that various cancer cells possess irregular G_1/S checkpoint controls, which induce these cells to an abnormal dependence of G_2 checkpoint mechanisms during cell cycle. Moreover, the regulation of G_2/M transition is more unpredictable in cancer cells than in normal cells.[32,33] In the last year, various studies led to believe G_2-phase transition as a crucial objective for the development of new antitumor drugs. Our data, gathered after **2j** treatments, suggested that this molecule could belong to this class of anticancer compounds.

Microtubules are a major component of the cytoskeleton involved in maintaining cell structure and cell division, including the formation of mitotic spindles, chromatid alignment and their division during mitosis. They are assembled by polymerization of alpha-beta dimers of tubulin. The polymerization is a reversible process, which is in a dynamic equilibrium. This phenomenon, known as microtubule dynamic instability, is a result of periods of rapid microtubule polymerization alternate with periods of shrinkage. Interference with the dynamics of microtubules polymerization and depolymerization, and consequently with cell division, has been proven to be clinically useful for anticancer drugs.[34] These drugs can either stabilize the polymerization process or destabilize it; in both cases the microtubule dynamics is disturbed, leading cell cycle to arrest and to apoptosis. Therefore, owing to their essential role in forming dynamic spindle apparatus in mitosis, microtubules are considered an ideal target for anticancer drug development.

Our experiments demonstrated a modification of β-tubulin filamentous and tubulin organization with abnormal distribution of tubulin around the nuclei, micronucleated cells and apoptotic and

pyknotic nuclei. Based on our data it is possible to assume that **2j** plays a key role in the microtubule assembly and dynamics.

One noteworthy feature occurring in cancer cells upon **2j** treatment, as evidenced by western blot, is an upregulation of p53. The p53 tumor suppressor protein is a transcription factor that responds to a variety of cellular stress conditions and controls key cellular processes, such as DNA damage and repair, hypoxia, cell cycle progression, differentiation, senescence, angiogenesis, metastasis and apoptosis.[35] p53 plays an important role in the arrest of damaged cells, favoring their repair and survival, or inducing cell cycle arrest and apoptosis when DNA is damaged irreparably.[36,37]

In our model, up-regulation of p53 seems to have a key role in mediating both anti-proliferative and pro-apoptotic effects of **2j**, suggesting that the deregulation of p53 and the G_2/M arrest could be linked to apoptosis by activation of apoptotic pathways or inhibition of cell cycle regulators via p53-dependent pathways.

All these data deliver interesting evidences and useful suggestion to speculate on the mode of action of **2j** focusing on targeted experiments in order to verify its molecular mechanisms.

Moreover, p53 could also induce cyclin-dependent kinase inhibitors, particularly Cdk1/Cyclin B complex, which plays a crucial role during G_2 phase. p53 transcriptionally represses Cyclin B_1, and Cdk1, but also Cyclin B_2, and Cyclin A inducing cell growth inhibition and G_2/M cell cycle arrest.[38, 39]

Furthermore, p53 could inhibit Topoisomerase, which plays a key role on chromosome segregation and progression into mitosis.[40] Topoisomerase II has a crucial function during the G_2/M transition, where it helps to form highly condensed mitotic chromosomes.[41] Moreover **2j** could induce a downregulation of topoisomerase II promoter activity, p53 dependent, blocking entry into mitosis.

Our findings underscore that oxadiazole derivatives could be interesting candidates for potential anticancer drugs and distinctly demonstrate that **2j** has robust antiproliferative activity, inhibits drastically growth and colony formation, and cause G_2/M cell cycle arrest and apoptosis, preferentially targeting cancer cells. Although further researches are ongoing to more fully elucidate

molecular targets affected by **2j,** additional investigations are fundamental to verify clinical evidences and explore its anticancer activity *in vivo*. In conclusion, based on our data, we affirm that **2j** is a prospective compound that could be evaluated as a promising candidate for new therapeutic strategies against cancer.

EXPERIMENTAL SECTION

Chemistry: All reactions involving air or moisture-sensitive compounds were performed under argon atmosphere. Solvents and reagents were obtained from commercial suppliers and were used without further purification. Microwave irradiation experiments were carried out in a Biotage® Microwave Initiator Eight 2.5 in the standard configuration as delivered, including proprietary software. All experiments were carried out in sealed microwave process vials under normal or low absorption. After completion of the reaction, the vial was cooled down to 25 °C via air jet cooling before opening. Reaction temperatures were monitored by an IR sensor on the outside wall of the reaction. Hydrogenations were carried out in the 4560 Parr Apparatus using a H2PEM-100 Parker Balston Hydrogen Generator. Flash column chromatography (FC) was performed automatically on Flash-master (Biotage®) with pre-packed Biotage® SNAP silica gel cartridges or manually on silica gel (Kieselgel 60, 0.040e0.063 mm, Merck®). The progress of all reactions was monitored by thin layer chromatography (TLC) performed with Polygram SIL N-HR/HV254 pre-coated plastic sheets (0.2 mm) on aluminum sheets (Kieselgel 60 F254, Merck®). Melting points were obtained on a Kofler melting point apparatus and are uncorrected. IR spectra were recorded as nujol mulls on NaCl plates with a Jasco FT/IR 460 plus spectrophotometer and are expressed in n (cm 1). NMR experiments were run on a Varian Unity 200 spectrometer (200.07 MHz for 1H, and 50.31 MHz for 13C) and Bruker Advance III spectrometer (400 MHz for 1H, and 101 MHz for 13C). Spectra were acquired using deuterated chloroform (chloroform-d) as solvent. Chemical shifts (d) for 1H and 13C NMR spectra are reported in parts per million (ppm) using the residual non-deuterated solvent resonance as the internal standard (for chloroform-d: 7.26 ppm, 1H and 77.16 ppm, 13C; for

DMSO-d6: 2.50 ppm, 1H, 39.52 ppm, 13C). Data are reported as follows: chemical shift (sorted in descending order), multiplicity (s for singlet, bs for broad singlet, d for doublet, t for triplet, q for quadruplet, m for multiplet), integration and coupling constants (J) in Hertz (Hz). Gas chromatography data was obtained using an Agilent 6850 FID gas chromatograph equipped with a HP-5 (5%-phenyl)-methylpolysiloxane capillary column (Agilent).

Purity of compounds. All final compounds purity was determined by elemental analysis on a PerkineElmer 240- B analyser, for C, H, and N. All of the final compounds were found to be >95% when analyzed.

General Procedure for the synthesis of Oxadiazoles

General Procedure A (2a, 2c, 2h, 2k, 2l). A solution of dihydrazide (**4a**, **4c**, **4h**, **9b-d**) (0.770 mmol) and $POCl_3$ (0.72 mL, 7.70 mmol, 10 eq) was refluxed at 110 °C for 2 h. The reaction mixture was concentrated under vacuum and carefully quenched with 5% $KHCO_3$ solution. The resulting precipitate was collected by filtration, dried and purified as indicated below to afford **2a**, **2c**, **2h**, **2k**, **2l** as solids.

General Procedure B (2b, 2d-g, 2i, 2o, 2p). To a solution of dihydrazide (**4b**, **4d-g**, **4i**, **9d**, **12a**) (0.650 mmol) in CH_3CN (7.8 mL) were added TEA (0.236 mL, 1.690 mmol, 2.6 eq), PPh_3 (0.341 g, 1.30 mmol, 2.0 eq) and CCl_4 (0.34 mL, 2.60 mmol, 4.0 eq). The reaction mixture was stirred at room temperature for 6-48 h and the precipitate was filtered and washed with ethanol and hexane to give **2b**, **2d-g**, **2i, 2o** and **2p** as solids.

General Procedure C (2j, 2q). To a suspension of hydrazide (**8a**, **11b**) (0.827 mmol) in DMF (1.7 mL) was added $PhC(OEt)_3$ (0.204 g, 0.910 mmol, 1.1 eq) and the mixture was stirred at 153 °C for 3-24 h. After that time, reaction was quenched pouring the solution into ice/water. The precipitate obtained was collected by filtration and purified as described below, affording **2j** and **2q** as solids.

General Procedure D (2m,n). Alkylboronic acid (0.275 mmol, 1.8 eq), K_3PO_4 (0.130 g, 0.612 mmol, 4 eq), $Pd(OAc)_2$ (0.005 g, 0.023 mmol, 0.15 eq) and PCy_3 (0.013 g, 0.046 mmol, 0.3 eq)

were added to a suspension of **2k** (0.060 g, 0.153 mmol) in toluene/H_2O (19:1, 3 mL) in a microwave vial. The mixture was degassed with argon for 10 min and warmed to 130 °C for 5 h in a microwave reactor. Solvent was removed under reduced pressure and the crude material was purified by flash chromatography with 30% of EtOAc in EtPet.

2-(4-Chlorophenyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2a). The title compound was prepared from **4a** following the general procedure A to afford **2a** as a white solid after flash chromatography with 40% of EtPet in EtOAc (37%), mp 277-278 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.17 (s, 1H), 8.10 (d, J = 8.2 Hz, 2H), 7.80 – 7.76 (m, 1H), 7.71 (d, J = 8.2 Hz, 2H), 7.54 (d, J = 7.4 Hz, 1H), 7.49 (d, J = 7.4 Hz, 1H), 7.31 (t, J = 7.4 Hz, 1H), 7.15 (d, J = 7.4 Hz, 1H), 3.76 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.2, 161.4, 146.3, 138.9, 136.2, 134.6, 129.6, 128.1, 126.6, 126.4, 125.5, 123.7, 122.6, 116.8, 103.5, 99.5, 30.6. GC-MS *m/z* 333.70 (M^+). Anal. Calcd for $C_{19}H_{12}ClN_3O$: C, 68.37; H, 3.62; Cl, 10.62; N, 12.59; O, 4.79. Found: C, 68.39; H, 3.61; N, 12.63.

2-(3-Chlorophenyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2b). The title compound was prepared from **4b** following the general procedure B to afford **2b** as a white solid (52%), mp 280-282 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.18 (s, 1H), 8.09 (d, J = 1.8 Hz, 1H), 8.06 (dd, J = 7.4, 1.2 Hz, 2H), 7.81 (d, J = 2.6 Hz, 1H), 7.74 – 7.63 (m, 2H), 7.55 (d, J = 7.4 Hz, 1H), 7.48 (d, J = 7.4 Hz, 1H), 7.31 (t, J = 7.4 Hz, 1H), 7.15 (t, J = 7.4 Hz, 1H), 3.76 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.4, 161.0, 146.4, 138.9, 134.63, 134.1, 131.4, 131.4, 126.6, 126.4, 125.8, 125.7, 125.5, 125.0, 124.0, 123.8, 116.8, 103.4, 30.6. GC-MS *m/z* 333.70 (M^+). Anal. Calcd for $C_{19}H_{12}ClN_3O$: C, 68.37; H, 3.62; Cl, 10.62; N, 12.59; O, 4.79. Found: C, 68.38; H, 3.65; N, 12.60.

2-(3,4-Dichlorophenyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2c). The title compound was prepared from **4c** following the general procedure A to afford **2c** as a grey solid after flash chromatography with 40% of EtPet in EtOAc (37%), mp 285-286 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.19 (s, 1H), 8.27 (d, J = 1.8 Hz, 1H), 8.05 (dd, J = 8.4, 1.8 Hz, 1H), 7.90 (d, J

= 8.4 Hz, 1H), 7.82 (d, *J* = 2.8 Hz, 1H), 7.54 (d, *J* = 7.4 Hz, 1H), 7.48 (d, *J* = 7.4 Hz, 1H), 7.31 (d, *J* = 7.4 Hz, 1H), 7.15 (t, *J* = 7.4 Hz, 2H), 3.76 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 162.5, 160.4, 146.3, 138.9, 134.6, 134.2, 132.3, 131.8, 127.8, 126.6, 126.4, 126.4, 125.5, 124.2, 124.1, 123.7, 116.8, 103.3, 30.6. GC-MS *m/z* 368.20 (M^+). Anal. Calcd for $C_{19}H_{11}Cl_2N_3O$: C, 61.98; H, 3.01; Cl, 19.25; N, 11.41; O, 4.34. Found: C, 62.01; H, 3.05; N, 11.44.

2-(4-(tert-Butil)phenyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2d). The title compound was prepared from **4d** following the general procedure B to afford **2d** as a white solid (46%), mp 289-291 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.15 (s, 1H), 7.81 (d, J = 2.2 Hz, 1H), 7.54 (d, J = 7.4 Hz, 1H), 7.49 (d, J = 7.4 Hz, 1H), 7.35 (s, 2H), 7.31 (d, J = 7.4 Hz, 1H), 7.14 (t, J = 7.4 Hz, 1H), 3.92 (s, 6H), 3.77 (s, 3H), 3.75 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.2, 161.8, 154.4, 146.3, 138.8, 134.7, 126.7, 126.4, 126.2, 126.1, 125.5, 123.7, 123.5, 121.0, 116.8, 103.7, 34.8, 30.8, 30.6. GC-MS *m/z* 355.40 (M^+). Anal. Calcd for $C_{23}H_{21}N_3O$: C, 77.72; H, 5.96; N, 11.82; O, 4.50. Found: C, 77.80; H, 5.98; N, 11.89.

2-(3,4,5-Trimethoxyphenyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2e). The title compound was prepared from **4e** following the general procedure B to afford **2e** as a white solid (60%), mp 265-266 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.80 (s, 1H), 7.53 (d, *J* = 6.2 Hz, 1H), 7.48 (d, *J* = 6.2 Hz, 1H), 7.40 – 7.23 (m, 3H), 7.14 (t, *J* = 7.6 Hz, 1H), 3.92 (s, 6H), 3.84 – 3.67 (m, 5H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.1, 161.9, 153.5, 146.4, 140.3, 138.9, 134.7, 126.6, 125.5, 123.7, 116.8, 103.8, 103.6, 60.2, 56.2, 30.6. GC-MS *m/z* 389.40 (M^+). Anal. Calcd for $C_{22}H_{19}N_3O_4$: C, 67.86; H, 4.92; N, 10.79; O, 16.43. Found: C, 67.95; H, 4.90; N, 10.74.

2-Cyclohexyl-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2f). The title compound was prepared from **4f** following the general procedure B to afford **2f** as a grey solid (8%), mp 191-192 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 1H), 7.59 (d, *J* = 2.2 Hz, 1H), 7.51 (d, *J* = 7.4 Hz, 1H), 7.46 (d, *J* = 7.4 Hz, 1H), 7.29 (d, *J* = 7.4 Hz, 1H), 7.12 (d, *J* = 7.4 Hz, 1H), 3.65 (s, 2H), 3.03 – 2.88 (m, 1H), 2.05 (d, *J* = 11.2 Hz, 2H), 1.81 – 1.72 (m, 2H), 1.69 – 1.62 (m, 1H), 1.62 – 1.52 (m, 2H), 1.47 – 1.36 (m, 2H), 1.35 – 1.23 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 167.5,

161.4, 146.3, 138.6, 134.7, 126.5, 126.2, 125.5, 123.6, 122.9, 116.7, 103.9, 34.1, 30.5, 29.7, 25.2, 24.7. GC-MS *m/z* 305.30 (M^+). Anal. Calcd for $C_{19}H_{19}N_3O$: C, 74.73; H, 6.27; N, 13.76; O, 5.24. Found: C, 74.81; H, 6.30; N, 13.75.

2-Benzyl-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2g). The title compound was prepared from **4g** following the general procedure B to afford **2g** as a white solid (12%), mp 183-184 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 7.58 (d, J = 2.6 Hz, 1H), 7.51 (d, J = 7.4 Hz, 1H), 7.46 (d, J = 7.5 Hz, 1H), 7.38 (d, J = 4.4 Hz, 3H), 7.33 – 7.25 (m, 2H), 7.12 (t, J = 7.2 Hz, 1H), 4.30 (s, 2H), 3.63 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 163.4, 162.1, 146.2, 138.7, 134.8, 134.6, 128.8, 128.7, 127.1, 126.5, 126.1, 125.5, 123.7, 123.1, 116.7, 103.7, 30.7, 30.5. GC-MS *m/z* 313.30 (M^+). Anal. Calcd for $C_{20}H_{15}N_3O$: C, 76.66; H, 4.83; N, 13.41; O, 5.11. Found: C, 76.70; H, 4.86; N, 13.40.

2-(4-Chlorobenzyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2h). The title compound was prepared from **4h** following the general procedure A to afford **2h** as a yellow solid after flash chromatography with 40% of EtPet in EtOAc (58%), mp: 235-237 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 7.58 (s, 1H), 7.51 (d, J = 7.4 Hz, 1H), 7.48 – 7.38 (m, 5H), 7.29 (t, J = 7.4 Hz, 1H), 7.12 (d, J = 7.4 Hz, 1H), 4.32 (s, 2H), 3.63 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 163.1, 162.1, 146.3, 138.8, 134.7, 133.9, 131.9, 130.8, 128.7, 126.6, 126.1, 125.5, 123.7, 123.1, 116.8, 103.6, 30.6, 30.0. GC-MS *m/z* 347.80 (M^+). Anal. Calcd for $C_{20}H_{14}ClN_3O$: C, 69.07; H, 4.06; Cl, 10.19; N, 12.08; O, 4.60. Found: C, 69.17; H, 4.09; N, 12.13.

2-(4-Methylbenzyl)-5-(1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-1,3,4-oxadiazole (2i). The title compound was prepared from **4i** following the general procedure B to afford **2i** as a white solid (35%) mp 201-202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 7.57 (d, J = 2.6 Hz, 1H), 7.51 (d, J = 7.4 Hz, 1H), 7.46 (d, J = 7.4 Hz, 1H), 7.31 – 7.26 (m, 1H), 7.25 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H), 7.12 (td, J = 7.4, 1.0 Hz, 1H), 4.24 (s, 2H), 3.63 (s, 2H), 2.28 (s, 3H).

^{13}C NMR (101 MHz, DMSO) δ 163.5, 162.0, 146.3, 138.7, 136.2, 134.7, 131.8, 129.3, 128.6, 126.5, 126.1, 125.5, 123.6, 123.0, 116.7, 103.7, 30.5, 30.3, 20.6. GC-MS *m/z* 327.10 (M^+). Anal. Calcd for $C_{21}H_{17}N_3O$: C, 77.04; H, 5.23; N, 12.84; O, 4.89. Found: C, 77.02; H, 5.26; N, 12.90.

2-(2-Methyl-1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole (2j). The title compound was prepared from **8a** following the general procedure C to afford **2j** as a white solid after flash chromatography with 50% of EtPet in EtOAc (76%), mp 257-258 °C. ^{1}H NMR (400 MHz, TFA-*d*) δ 7.99 – 7.81 (m, 1H), 7.67 – 7.58 (m, 1H), 7.58 – 7.47 (m, 2H), 7.35 – 7.24 (m, 1H), 7.20 – 7.11 (m, 1H), 7.12-7.09 (m, 1H), 7.08 – 6.98 (m, 1H), 6.99 – 6.82 (m, 1H), 3.49 (s, 2H), 2.58 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$/TFA-*d*) δ 163.1, 161.3, 145.8, 144.2, 144.1, 140.1, 135.1, 133.0, 130.2, 127.4, 125.8, 125.7, 120.1, 117.4, 96.0, 31.3, 14.4. GC-MS *m/z* 313.30 (M^+). Anal. Calcd for $C_{20}H_{15}N_3O$: C, 76.66; H, 4.83; N, 13.41; O, 5.11. Found: C, 76.72; H, 4.85; N, 13.44.

2 -(6-Bromo-2-methyl-1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole (2k). The title compound was prepared from **9b** following the general procedure A to afford **2k** as a beige solid after flash chromatography with 50% of EtPet in EtOAc (64%), mp Over 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.02 (s, 1H), 8.11 – 7.98 (m, 2H), 7.68 (s, 1H), 7.66 – 7.58 (m, 3H), 7.53 – 7.40 (m, 1H), 7.30 (d, J = 8.0 Hz, 1H), 3.76 (s, 2H), 2.69 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.4, 161.8, 148.4, 135.7, 135.3, 134.1, 131.5, 129.4, 129.3, 128.3, 127.8, 126.2, 123.8, 117.5, 115.7, 100.9, 31.0, 13.6. GC-MS *m/z* 392.30 (M^+). Anal. Calcd for $C_{20}H_{14}BrN_3O$: C, 61.24; H, 3.60; Br, 20.37; N, 10.71; O, 4.08. Found: C, 61.33; H, 3.68; N, 10.82.

2-(6-Chloro-2-methyl-1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole (2l). The title compound was prepared from **9c** following the general procedure A to afford **2l** as a beige solid after flash chromatography with 50% of EtPet in EtOAc (61%), mp Over 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.00 (s, 1H), 8.08 – 8.04 (m, 2H), 7.64 – 7.60 (m, 3H), 7.54 (s, 1H), 7.34 – 7.32 (m, 2H), 3.75 (s, 2H), 2.68 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.4, 161.8, 148.0, 135.5, 135.2, 133.7, 131.5, 129.4, 127.8, 127.5, 126.5, 126.2, 125.5, 123.8, 116.9, 100.9, 31.0, 13.6. GC-

MS *m/z* 347.80(M^+). Anal. Calcd for $C_{20}H_{14}ClN_3O$: C, 69.07; H, 4.06; Cl, 10.19; N, 12.08; O, 4.60. Found: C, 69.22; H, 4.03; N, 12.57.

2-(2,6-Dimethyl-1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole (2m). The title compound was prepared from **2k** following the general procedure D to afford **2m** as a white solid after flash chromatography with 50% of EtPet in EtOAc (34%), mp 224-225 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.89 (s, 1H), 8.11 – 8.00 (m, 2H), 7.66 – 7.58 (m, 3H), 7.31 (s, 1H), 7.26 (d, J = 6.6 Hz, 1H), 7.08 (d, J = 6.6 Hz, 1H), 3.69 (s, 2H), 2.68 (s, 3H), 2.33 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.6, 161.7, 146.1, 136.3, 134.4, 132.4, 132.1, 131.4, 129.4, 126.9, 126.4, 126.3, 126.2, 123.8, 115.7, 100.7, 30.8, 21.1, 13.5. GC-MS *m/z* 327.40 (M^+). Anal. Calcd for $C_{21}H_{17}N_3O$: C, 77.04; H, 5.23; N, 12.84; O, 4.89. Found: C, 77.34; H, 5.25; N, 13.01.

2-(6-Cyclopropyl-2-methyl-1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole (2n). The title compound was prepared from **2k** following the general procedure D to afford **2n** as a white solid after flash chromatography with 50% of EtPet in EtOAc (50%), mp 206-208 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H), 8.07 (dd, J = 3.0, 6.6 Hz, 2H), 7.69 – 7.58 (m, 3H), 7.25 (d, J = 7.8 Hz, 1H), 7.21 (s, 1H), 7.00 (d, J = 7.8 Hz, 1H), 3.68 (s, 2H), 2.68 (s, 3H), 1.93 (tt, J = 5.0, 8.8 Hz, 1H), 1.03 – 0.72 (m, 2H), 0.70 – 0.64 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 162.6, 161.7, 146.2, 138.6, 136.2, 134.4, 132.4, 131.4, 129.4, 126.4, 126.2, 123.8, 122.6, 115.7, 100.7, 39.5, 30.9, 15.2, 13.5, 9.1. GC-MS *m/z* 353.40 (M^+). Anal. Calcd for $C_{23}H_{19}N_3O$: C, 78.16; H, 5.42; N, 11.89; O, 4.53. Found: C, 78.77; H, 5.49; N, 11.95.

2-(7-Methoxy-2-methyl-1,4-dihydroindeno[1,2-b]pyrrol-3-yl)-5-phenyl-1,3,4-oxadiazole (2o). The title compound was prepared from **9d** following the general procedure B to afford **2o** as a light yellow solid after flash chromatography with 50% of EtPet in EtOAc (61%)mp 231-233 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.93 (s, 1H), 8.07 (dd, J = 3.0, 6.6 Hz, 2H), 7.84 – 7.55 (m, 3H), 7.39 (d, J = 8.2 Hz, 1H), 6.96 (d, J = 2.4 Hz, 1H), 6.66 (dd, J = 2.4, 8.2 Hz, 1H), 3.79 (s, 3H), 3.67 (s, 2H), 2.69 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.5, 161.7, 158.6, 137.6, 136.0, 135.0, 131.4, 129.4, 128.4, 126.2, 125.7, 123.8, 108.2, 102.5, 100.8, 55.1, 30.2, 13.6. GC-MS *m/z* 343.40

(M^+). Anal. Calcd for $C_{21}H_{17}N_3O_2$: C, 73.45; H, 4.99; N, 12.24; O, 9.32. Found: C, 73.98; H, 5.07; N, 12.65.

2-(1-Methyl-1,4-dihydroindeno[1,2-c]pyrazol-3-yl)-5-phenyl-1,3,4-oxadiazole (2p). The title compound was prepared from **12a** following the general procedure B to afford **2p** as a white solid (22%), mp 262-264 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.11 (d, J = 1.6 Hz, 1H), 8.09 (d, J = 2.4 Hz, 1H), 7.86 (d, J = 7.4 Hz, 1H), 7.70 – 7.61 (m, 4H), 7.45 (d, J = 7.2 Hz, 1H), 7.38 (d, J = 7.2 Hz, 1H), 4.26 (s, 3H), 3.89 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.3, 159.7, 150.0, 148.4, 132.0, 130.8, 129.7, 129.5, 127.0, 126.8, 126.6, 126.5, 123.3, 119.4, 38.4, 29.0. GC-MS *m/z* 314.30 (M^+). Anal. Calcd for $C_{19}H_{14}N_4O$: C, 72.60; H, 4.49; N, 17.82; O, 5.09. Found: C, 72.70; H, 4.45; N, 17.89.

2-(1-Phenyl-1,4-dihydroindeno[1,2-c]pyrazol-3-yl)-5-phenyl-1,3,4-oxadiazole (2q). The title compound was prepared from **11b** following the general procedure C to afford **2q** as a white solid after flash chromatography with 15% of EtOAc in EtPet (45%), mp 248-249 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.15 (dd, J = 7.6, 1.9 Hz, 1H), 7.91 (d, J = 7.8 Hz, 1H), 7.78 – 7.61 (m, 8H), 7.58 – 7.40 (m, 3H), 7.23 (d, J = 7.0 Hz, 1H), 3.29 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.2, 158.0, 157.9, 139.5, 137.8, 134.1, 132.5, 132.5, 131.8, 131.0, 130.0, 130.0, 129.6, 126.8, 124.8, 124.0, 122.9, 121.7, 120.4, 29.1. GC-MS *m/z* 376.40 (M^+). Anal. Calcd for $C_{24}H_{16}N_4O$: C, 76.58; H, 4.28; N, 14.88; O, 4.25. Found: C, 76.66; H, 4.30; N, 14.95.

General Procedure for the synthesis of Dihydrazides

General Procedure E (4a, 4c, 4f, 4g, 9b-d). To a solution of **3** or **8b-d** (0.940 mmol) in *N*-methyl-2-pyrrolidone (5 mL) was added dropwise the required acyl chloride (0.940 mmol, 1.0 eq). After stirring the reaction for 24 h at room temperature, the mixture was poured into ice/water and 5% $KHCO_3$ solution was added until pH 7. The precipitate was filtered, washed with water and dried to afford the corresponding products **4a**, **4c**, **4f**, **4g**, **9b-d**.

General Procedure F (4b, 4d, 4e, 12a). To a suspension of 1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**3**) (0.200 g, 0.940 mmol) in DCM (5 mL) was added dropwise the required acyl

chloride (1.175 mmol, 1.25 eq) and pyridine (0.094 mmol, 0.1 eq,). After stirring at room temperature for 2-24 h, the suspension was filtered and washed with water. The analytically pure products **4b**, **4d**, **4e** were isolated by flash chromatography as indicated below.

General Procedure G (4h, 4i). To a suspension of 1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**3**) (0.200 g, 0.940 mmol) in DCM/DMF (1:1 10 mL) were added the corresponding benzyl carboxylic acid (1.034 mmol, 1.1 eq), EDCI (1.128 mmol, 1.5 eq) HOBt (1.880 mmol, 2 eq) and NMM (2.820 mmol, 3 eq). The mixture was stirred at room temperature for 24 h, then quenched with HCl 1.5 M. The precipitate was filtered, washed with water and dried to afford the corresponding dihydazides **4h-i** as white solids.

N'-(4-Chlorobenzoyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4a). The title compound was prepared from **3** and 4-chlorobenzoyl chloride using the general procedure E to afford **4a** as a white solid (0.201 g, 61%), mp 260-262 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.79 (s, 1H), 10.43 (s, 1H), 9.73 (s, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.58 (d, J = 2.2 Hz, 1H), 7.47 (d, J = 7.4 Hz, 1H), 7.42 (d, J = 7.4 Hz, 1H), 7.27 (t, J = 7.4 Hz, 1H), 7.09 (t, J = 7.4 Hz, 1H), 3.62 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.0, 163.6, 146.4, 137.7, 136.5, 134.9, 131.6, 129.3, 128.6, 127.9, 126.4, 125.3, 123.9, 123.3, 116.3, 113.6, 30.8.

N'-(3-Chlorobenzoyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4b). The title compound was prepared from **3** and 4-(*tert*-butyl)benzoyl chloride using the general procedure F to afford **4b** after 24 h as a light green solid after flash chromatography with 30% of EtPet in EtOAc (0.255 g, 77%), mp 264-265 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.81 (s, 1H), 10.48 (s, 1H), 9.78 (s, 1H), 7.96 (s, 1H), 7.89 (d, J = 7.2 Hz, 1H), 7.67 (d, J = 7.8 Hz, 1H), 7.61 – 7.51 (m, 2H), 7.47 (d, J = 7.2 Hz, 1H), 7.43 (d, J = 7.2 Hz, 2H), 7.27 (t, J = 7.2 Hz, 2H), 7.09 (d, J = 7.2 Hz, 1H), 3.63 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.7, 163.7, 146.5, 137.8, 134.9, 134.8, 133.4, 131.6, 130.6, 127.9, 127.3, 126.5, 126.2, 125.4, 124.0, 123.3, 116.4, 113.6, 39.5, 30.9.

N'-(3,4-Dichlorobenzoyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4c). The title compound was prepared from **3** and 3,4-dichlorobenzoyl chloride using the general procedure E to

afford **4c** as a white solid (0.224 g, 62%), mp 250-252 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.81 (s, 1H), 10.54 (s, 1H), 9.79 (s, 1H), 8.15 (s, 1H), 7.91 (d, J = 8.8 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.58 (s, 1H), 7.47 (d, J = 8.0 Hz, 1H), 7.42 (d, J = 7.2 Hz, 1H), 7.27 (t, J = 7.6 Hz, 1H), 7.09 (t, J = 7.6 Hz, 1H), 3.62 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.9, 163.6, 146.5, 137.8, 134.9, 134.6, 133.1, 131.5, 131.0, 129.4, 127.7, 126.4, 125.4, 124.0, 123.3, 116.4, 113.5, 30.8.

N'-(4-(tert-Butyl)benzoyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4d). The title compound was prepared from **3** and 4-(*tert*-butyl)benzoyl chloride using the general procedure F to afford **4d** after 24 h as a white solid after flash chromatography with 30% of EtPet in EtOAc (0.222 g, 63%), mp 264-265 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (s, 1H), 10.25 (s, 1H), 9.68 (s, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.58 (s, 1H), 7.53 (d, J = 8.0 Hz, 2H), 7.47 (d, J = 7.6 Hz, 1H), 7.42 (d, J = 7.6 Hz, 1H), 7.27 (t, J = 7.5 Hz, 1H), 7.09 (t, J = 7.5 Hz, 1H), 3.62 (s, 2H), 1.32 (s, 7H). ^{13}C NMR (101 MHz, DMSO- d_6) □□ 165.9, 163.7, 154.5, 146.5, 137.7, 134.9, 130.1, 127.9, 127.3, 126.4, 125.3, 125.2, 123.8, 123.2, 116.3, 113.8, 99.5, 39.5, 34.7, 30.9, 30.8.

N'-(3,4,5-Trimethoxybenzoyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4e). The title compound was prepared from **3** and 4-(*tert*-butyl)benzoyl chloride using the general procedure F after 24 h to afford **4e** as a white solid after flash chromatography with 10% of EtPet in EtOAc (0.140 g, 36%), mp 219-220 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.79 (s, 1H), 10.29 (s, 1H), 9.70 (s, 1H), 7.59 (s, 1H), 7.47 (d, J = 7.4 Hz, 1H), 7.42 (d, J = 7.4 Hz, 1H), 7.29 – 7.22 (m, 3H), 7.09 (t, J = 7.4 Hz, 1H), 3.85 (s, 6H), 3.74 (s, 3H), 3.63 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.4, 163.7, 152.7, 146.5, 140.3, 137.7, 134.9, 127.9, 126.4, 125.4, 123.9, 123.3, 116.4, 113.7, 105.0, 60.1, 56.0, 30.9.

N'-(Cyclohexanecarbonyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4f). The title compound was prepared from **3** and cyclohexanecarbonyl chloride using the general procedure E to afford **4f** as a white solid (0.243 g, 81%), mp 180-182 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ 11.72 (s, 1H), 9.58 (s, 1H), 9.46 (s, 1H), 7.52 (d, J = 2.6 Hz, 1H), 7.46 (d, J = 7.4 Hz, 2H), 7.40 (d, J = 7.4 Hz, 1H), 7.25 (d, J = 7.4 Hz, 1H), 7.07 (t, J = 7.4 Hz, 1H), 3.58 (s, 2H), 2.24 (t, J = 11.4 Hz, 1H),

1.78 – 1.69 (m, 4H), 1.67 – 1.59 (m, 1H), 1.46 – 1.33 (m, 2H), 1.32 – 1.14 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 174.7, 163.3, 146.5, 137.6, 134.9, 127.9, 126.4, 125.3, 123.7, 123.2, 116.3, 113.8, 42.1, 30.8, 29.1, 25.4, 25.2.

N'-(2-Phenylacetyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4g). The title compound was prepared from **3** and 2-phenylacetyl chloride using the general procedure E to afford **4g** as a white solid (0.192 g, 61%), mp 146-147 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.75 (s, 1H), 10.00 (s, 1H), 9.56 (s, 1H), 7.52 (s, 1H), 7.45 (d, J = 7.2 Hz, 1H), 7.40 (d, J = 7.6 Hz, 1H), 7.38 – 7.30 (m, 4H), 7.28 – 7.22 (m, 2H), 7.07 (t, J = 7.2 Hz, 1H), 3.59 (s, 2H), 3.52 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.6, 163.4, 146.5, 137.7, 135.9, 134.9, 129.1, 128.2, 127.9, 126.5, 126.4, 125.4, 123.9, 123.3, 116.4, 113.6, 40.4, 30.9.

N'-(2-(4-Chlorophenyl)acetyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4h). The title compound was prepared from **3** and 2-(4-chlorophenyl)acetic acid using the general procedure G to afford **4h** as a white solid (0.225 g, 66%), mp 235-236 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.75 (s, 1H), 10.02 (s, 1H), 9.58 (s, 1H), 7.56 – 7.49 (m, 1H), 7.48 – 7.30 (m, 7H), 7.30 – 7.21 (m, 1H), 7.12 – 7.02 (m, 1H), 3.59 (s, 2H), 3.53 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 163.3, 146.4, 137.6, 134.9, 131.2, 130.9, 128.1, 127.8, 126.4, 125.3, 123.8, 123.2, 116.3, 113.5, 40.4, 30.8.

N'-(2-(4-Methylphenyl)acetyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (4i). The title compound was prepared from **3** and 2-(4-methylphenyl)acetic acid using the general procedure G to afford **4i** as a white solid (0.161 g, 51%), mp 201-202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.74 (s, 1H), 9.96 (s, 1H), 9.54 (s, 1H), 7.52 (d, J = 2.2 Hz, 1H), 7.45 (d, J = 7.4 Hz, 1H), 7.40 (d, J = 7.4 Hz, 1H), 7.28 – 7.19 (m, 3H), 7.12 (d, J = 7.8 Hz, 2H), 7.07 (t, J = 7.5 Hz, 1H), 3.59 (s, 2H), 3.46 (s, 2H), 2.28 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.7, 163.3, 146.5, 137.6, 135.4, 134.9, 132.8, 128.9, 128.7, 127.9, 126.4, 125.3, 123.8, 123.2, 116.3, 113.6, 40.2, 30.8, 20.7.

N'-Benzoyl-6-bromo-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (9b). The title compound was prepared from **8b** and benzoyl chloride using the general procedure E to afford **9b** as a white solid (0.366g, 95%), mp 157-159 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.72 (s, 1H),

10.33 (s, 1H), 9.23 (s, 1H), 7.97 – 7.86 (m, 2H), 7.63 (s, 1H), 7.58 – 7.53 (m, 1H), 7.53 – 7.47 (m, 2H), 7.43 (d, J = 7.8 Hz, 1H), 7.26 (dd, J = 7.9, 2.6 Hz, 1H), 3.75 (s, 2H), 2.53 (s, 3H). ^{13}C NMR(101 MHz, DMSO*)* δ 165.5, 164.4, 148.3, 137.0, 134.3, 134.2, 133.7, 131.3, 129.2, 128.3, 128.0, 127.4, 127.3, 117.1, 115.2, 110.2, 31.2, 13.4.

N'-Benzoyl-6-chloro-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (9c). The title compound was prepared from **8c** and benzoyl chloride using the general procedure E to afford **9c** as a white solid (0.296 g, 86%), mp 164-166 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.73 (s, 1H), 10.35 (s, 1H), 9.16 (s, 1H), 7.94 (d, J = 7.2 Hz, 2H), 7.62 – 7.56 (m, 1H), 7.55 – 7.47 (m, 3H), 7.32 – 7.27 (m, 2H), 3.76 (s, 2H), 2.53 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.8, 164.9, 148.0, 137.1, 133.9, 133.8, 132.9, 131.7, 128.5, 127.5, 127.4, 127.1, 126.4, 125.3, 116.7, 110.1, 31.3, 13.4.

N'-Benzoyl-7-methoxy-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (9d). The title compound was prepared from **3** and 2-phenylacetyl chloride using the general procedure E to afford **9d** as a white solid (0.241 g, 71%), mp 198-200 °C. ^{1}H NMR ^{1}(200 MHz, DMSO-d_6) δ 10.94 (s, 1H), 10.09 (s, 1H), 8.37 (s, 1H), 8.04 (d, J = 6.8 Hz, 1H), 7.98 (d, J = 6.8 Hz, 1H), 7.40 – 7.54 (m, 3H), 7.30 (d, J = 8.0 Hz, 1H), 6.97 (d, J = 2.4Hz, 1H), 6.61 (dd, J = 8.0, 2.4 Hz, 1H), 3.84 (s, 3H), 3.76 (s, 2H), 2.53 (s, 3H).

N'-Benzoyl-1-methyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (12a). The title compound was prepared from **11a** and benzoyl chloride using the general procedure F to afford **12a** after 2 h as a white solid (0.249 g, 95%), mp 168-169 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (s, 1H), 10.06 (s, 1H), 7.92 (d, J = 7.2 Hz, 2H), 7.81 (d, J = 7.6 Hz, 1H), 7.59 (t, J = 7.4 Hz, 2H), 7.52 (t, J = 7.3 Hz, 2H), 7.42 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 7.3 Hz, 1H), 4.20 (s, 3H), 3.72 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.6, 160.9, 149.6, 148.7, 138.0, 132.7, 131.7, 131.0, 128.4, 127.5, 127.3, 126.9, 126.5, 119.1, 39.5, 29.1.

General Procedure H for the synthesis of Tricyclic Esters (7a-d). To a solution of ethyl acetoacetate (0.290 g, 2.24 mmol, 1.1 eq) in THF (5 mL) at 0 °C was slowly added NaH 60% in mineral oil (0.095 g. 2.24 mmol, 1.1 eq). After 30 min the appropriate ketone **5a-d** (2.035 mmol) in

THF (5 mL) was dripped into the mixture and stirred overnight at room temperature. Reaction as quenched with water and extracted with DCM. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated in vacuo. Crude obtained was dissolved in EtOH (15 mL) and was reacted with NH_4OAc (0.470 g, 6.105 mmol, 3 eq) at 80 °C overnight. Solvent was removed in vacuo, mixture was dissolved in EtOAc and washed with water. Organic layer was dried and evaporated at reduce pressure to give an oil purified as described.

Ethyl 2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxylate (7a). The title compound was prepared from **5a** using the general procedure H to afford **7a** as orange solid (0.161 g, 92%) after flash chromatography with 15 % of EtOAc in EtPet, mp 177-179 °C. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 8.67 (s, 1H), 7.45 (d, J = 7.4 Hz, 1H), 7.24 (d, J = 6.8 Hz, 2H), 7.09 (t, J = 7.0 Hz, 1H), 4.32 (q, J = 7.1 Hz, 2H), 3.64 (s, 2H), 2.64 (s, 3H), 1.39 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$-*d*) δ 166.0, 146.7, 138.9, 135.1, 135.0, 130.8, 126.4, 125.4, 123.3, 115.8, 109.2, 59.6, 32.2, 14.7, 14.2.

Ethyl 6-bromo-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxylate (7b). The title compound was prepared from **5b** using the general procedure H to afford **7b** as beige solid (0.235 g, 36%) after crystallization with MeOH, mp: 210-211 °C. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 8.41 (s, 1H), 7.57 (s, 1H), 7.37 (dd, J = 8.0, 1.4 Hz, 1H), 7.10 (d, J = 8.0 Hz, 1H), 4.32 (q, J = 7.0 Hz, 2H), 3.63 (s, 2H), 2.65 (s, 3H), 1.39 (d, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.6, 148.7, 139.5, 134.4, 134.3, 130.9, 129.3, 128.5, 116.9, 116.5, 109.0, 77.2, 59.5, 32.1, 14.7, 14.1.

Ethyl 6-chloro-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxylate (7c). The title compound was prepared from **5c** using the general procedure H to afford **7c** as beige solid (0.252 g, 45%) after crystallization with MeOH, mp 203-205 °C. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 8.49 (s, 1H), 7.41 (s, 1H), 7.21 (d, J = 8.0 Hz, 1H), 7.14 (d, J = 8.0 Hz, 1H), 4.31 (q, J = 7.0 Hz, 2H), 3.62 (s, 2H), 2.64 (s, 3H), 1.39 (d, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.6, 148.4, 139.2, 134.2, 133.5, 131.1, 129.0, 126.5, 125.9, 116.3, 109.4, 77.2, 59.6, 32.2, 14.7, 14.2.

Ethyl 7-methoxy-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxylate (7d). The title compound was prepared from **5a** using the general procedure H to afford **7a** as yellow solid (27%)

after flash chromatography with 15 % of EtOAc in EtPet, mp 168-170 °C.^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 8.46 (s, 1H), 7.33 (d, J = 8.4, 1H), 6.83 (d, J = 2.4, 1H), 6.61 (dd, J = 8.4, 2.4 Hz, 1H), 4.31 (q, J = 7.0 Hz, 2H), 3.83 (s, 3H), 3.58 (s, 2H), 2.65 (s, 3H), 1.39 (d, J = 7.0 Hz, 3H).

General Procedure I for the synthesis of Hydrazides (8a-d, 11a,b). To a solution of ester **7a-d** or **11a**,**b** (1.5 mmol) in absolute EtOH (2.25 mL) hydrazine monohydrate 98% (60 mmol, 40 eq) was added and the reaction was refluxed stirred for 24 - 72 h. After that time, the mixture was poured into ice/water to give a precipitate that was filtered, washed with water and dried.

2-Methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (8a). The title compound was prepared from **7a** using the general procedure I to afford **8a** after 24 h as a light yellow solid (0.143 g, 42 %), mp 235-236 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.51 (s, 1H), 8.37 (s, 1H), 7.40 (d, J = 7.4 Hz, 1H), 7.29 (d, J = 7.4 Hz, 1H), 7.21 (d, J = 7.4 Hz, 1H), 7.01 (t, J = 7.4 Hz, 1H), 4.30 (s, 2H), 3.62 (s, 2H), 2.50 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 166.0, 145.7, 135.4, 135.1, 134.5, 126.4, 126.3, 125.0, 122.5, 115.6, 110.3, 39.5, 30.9, 13.3.

6-Bromo-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (8b). The title compound was prepared from **7b** using the general procedure I to afford **8b** after 72 h as a light yellow solid (0.436 g, 95 %), mp 266-268 °C ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (s, 1H), 8.43 (s, 1H), 7.59 (s, 1H), 7.41 (dd, J = 8.0, 1.4 Hz, 1H), 7.22 (d, J = 8.0 Hz, 1H), 4.30 (s, 2H), 3.66 (s, 2H), 2.50 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.8, 148.3, 136.1, 134.3, 133.5, 129.1, 127.9, 127.2, 117.1, 115.0, 110.5, 31.0, 13.3.

6-Chloro-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (8c). The title compound was prepared from **7c** using the general procedure I to afford **8c** after 72 h as a light yellow solid (0.350 g, 89 %), mp 255-256 °C^{1}H NMR (400 MHz, DMSO-d_6) δ 11.56 (s, 1H), 8.42 (s, 1H), 7.46 (s, 1H), 7.29 – 7.17 (m, 2H), 4.31 (s, 2H), 3.66 (s, 2H), 2.50 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.9, 148.0, 136.0, 134.0, 133.5, 127.2, 126.8, 126.3, 125.2, 116.5, 110.5, 31.0, 13.3.

7-Methoxy-2-methyl-1,4-dihydroindeno[1,2-b]pyrrole-3-carbohydrazide (8d). The title compound was prepared from **7d** using the general procedure I to afford **8d** after 48 h as a light yellow solid

(0.189 g, 49 %), mp 220-222 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.55 (s, 1H), 7.30 (d, J = 8.2 Hz, 1H), 7-.07 (s, 1H), 6.93 (d, J = 2.2 Hz, 1H), 6.60 (dd, J = 8.2, 2.2 Hz, 1H), 3.83 (s, 3H), 3.52 (s, 2H), 2.65 (s, 3H).

1-Methyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (11a). The title compound was prepared from **10a** using the general procedure I to afford **11a** as a light yellow solid (0.308 g, 90 %), mp 178-179 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (s, 1H), 7.76 (d, J = 7.4 Hz, 1H), 7.58 (d, J = 7.4 Hz, 1H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 4.39 (s, 2H), 4.12 (s, 3H), 3.68 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.2, 149.3, 148.6, 138.6, 131.1, 126.8, 126.4, 126.3, 119.0, 39.5, 29.0.

1-Phenyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (11b). The title compound was prepared from **10b** using the general procedure I to afford **11b** as a white solid (0.379 g, 87 %), mp 158-160°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.49 (s, 1H), 7.80 (d, J = 7.6 Hz, 2H), 7.69 – 7.62 (m, 3H), 7.54 (t, J = 7.2 Hz, 1H), 7.46 – 7.42 (m, 1H), 7.36 – 7.32 (m, 2H), 4.48 (s, 2H), 3.81 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.9, 149.2, 148.4, 140.8, 139.3, 130.9, 129.7, 128.4, 126.9, 126.9, 126.6, 123.2, 119.0, 29.0.

Biology

Cell culture medium and supplements.

Mcf7 (breast cancer), HeLa (cervix adenocarcinoma), RD (rhabdomyosarcoma), T98G (glioblastoma multiforme), A549, H1299 (non-small lung cancer), PC3 (prostate cancer) were purchased from ATCC; SH-SY5Y, Kelly, Lan1, SK-N-AS (neuroblastoma) were provided by Professor Arturo Sala, Brunel University, London. The cell lines were grown in Dulbecco's Modified Eagle's Medium (Gibco), containing 10% (v/v) Fetal Bovine Solution (FBS) (Gibco), 100 units/mL penicillin and 100 □g/mL streptomycin (Gibco). NB1, NB1 hTERT, Mrc5, Mrc5-SV1 S40, IBR3, IBR3-G SV40 cell lines, a kind gift from Dr. Cristopher Parris, Brunel University, London, were cultured in DMEM supplemented with 10% Fetal Calf Serum, 100 units/mL

penicillin and 100 □g/mL streptomycin and 1% L-glutamine.

All cell lines were cultured at 37 °C in 5% CO_2.

Proliferation assay (XTT).

Cell viability was measured using the XTT assay (Cell proliferation kit II, Roche): 0.5 □L XTT electron coupling reagent and 74.5 □L medium (final volume 100 □L/well) were added in each well and incubated for 4 h at 37 °C.

Cells were plated at a plating density ranging from 1500 to 2000 cells/well, depending on the doubling time of individual cell lines, on 96 well plates.

After 24 hours, different concentrations of molecules were added into six triplicate wells, and the cells were incubated for additional 72 hours without changing the medium, before the XTT assay was performed. The plates were shaken for 15 seconds, and then read at 490 nm with a spectrophotometric plate reader.

The cytotoxic effect on cell lines was expressed as the IC_{50} value (the drug concentration reducing the absorbance of treated cells by 50 % with respect to that of untreated cells). All experiments were carried out in triplicate.

Colony formation assay.

100-200 cells/well were seeded in a six-well plates and incubated at 37 °C for 2 to 3 weeks, until the cells in control dishes had formed visible colonies.

Cells were treated and the culture medium with the compounds was changed every 3 days. The cells were stained with 2–3 mL of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 30 min. The stained colonies were counted and compared with the control cells.

Cytotoxicity assay.

The cell lines were plated into 96-well plates. After 24 hours, the cells were treated with **2j** (0.5 □M), different concentrations of Harmine (1-3-5 □M) or Reversan (5-15-25 □M) and with the combination of **2j**-Harmine or 2j-Reversan and incubated for 72 hours at 37 °C and 5% CO_2. Then,

XTT solution was added in each well and incubated for 4 hours at 37 °C. The absorbance was read at 490 nm with a spectrophotometric plate reader.

Immunostaining.

1 x 10^5 cells/well were seeded on 13 mm glass coverslips in a 24 well plate. The cells were treated with 1 μM of **1a** and **2j** and, 24 hours later, the cells were washed and fixed using 4% paraformaldehyde (PFA) for 15 min. Cells were permeabilized with 0.5% Triton-X 100 for 10 min, blocked in standard blocking buffer (5% BSA in PBS) for 1 hours at room temperature and incubated with primary antibody *h*β-tubulin (Sigma T4026) 1:200 diluted in 1% BSA in PBS overnight at 4 °C. Coverslips were washed with 1X PBS and then incubated with secondary antibody (Alexa Fluor®) diluted in 1% BSA in PBS for 1 hours at RT. After washing in PBS, the slides were counterstained with DAPI (1mg/mL), diluted in PBS for 1 min at RT and cover-slipped using a MOVIOL (Calbiochem). Images were acquired using a microscope.

Flow Cytometry Analysis.

Cells were plated on 6 cm dishes and after 24 hours were treated with 1 □M of **1a** and **2j** for different times: 2-24 hours for HeLa, T98G and RD or 24-72 hours for Mcf7, SH-SY5Y, Kelly, Lan1, SK-N-AS, A549 and H1299.

Cells were centrifuged at 3000 rpm for 5 min, washed in PBS and fixed in 70 % ice-cold ethanol and incubated at -20°C overnight. Fixed cells were washed twice with PBS, and then incubated overnight under dark condition at 4 °C in a solution containing 20 □g/mL RNase A and 5 □g/mL iodide propidium (PI, Sigma Aldrich) in PBS. The flow cytometric analysis was performed using a flow cytometry (BD Accuri C6 Flow Cytometer) by collecting 20,000 events and the data were analyzed using the BD Accuri C6 software (BD Biosciences).

Apoptosis.

Annexin V-propidium iodide assay was performed, by using FITC Annexin V Apoptosis Detection Kit II (BD Pharmigen), according to the manufacturer's instructions.

Cells were treated with 5 □M of **2j** for 72 hours; adherent and floating cells were harvested, centrifuged and washed two times with cold PBS. The cells were resuspended in 1 mL binding buffer. Next, 5 □L Annexin-V-fluorescein isothiocyanate and 5 □L PI were added and incubated in the dark for 15 min at room temperature. Flow cytometry analysis was performed immediately after supravital staining.

Immunoblotting.

Cells were lysated in lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 10% glycerol 1% Nonidet P-40; 2 mM EDTA) supplemented with Protease Inhibitor Cocktails (Roche).

Equal amounts of lysates were resolved by 10% SDS/PAA gel and transferred to nitrocellulose membranes (GE Healthcare, Whatman) at 4 °C and at 100V for 1 hours.

Blots were then blocked for 1 hour in 5% non-fat dry milk powder in 1% TBS-T with gentle agitation and incubated overnight with the primary antibody p53 (Santa Cruz sc-126) and anti-GAPDH (Santa Cruz sc-25778) in 3% non-fat dry milk in 1X TBS-T. Membranes were then incubated with HRP-conjugated secondary antibodies anti-rabbit and anti-mouse (1:10.000) peroxidase conjugated (Santa Cruz).

Enhanced chemiluminescence was performed according to manufacturer's instructions (SuperSignal West Fempto Maximum Sensitivity Substrate, Thermo Scientific).

Statistical analysis. Data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical significance analysis was determined by using Student's two-tailed, unpaired t-test, for multiple comparisons with the control. The differences were considered significant for p values less than 0.05.

ASSOCIATED CONTENT

The Supporting Information is available

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ABBREVIATION USED

EDCI: 1-Ethil-3-(3-dimethylaminopropyl)carbodiimide, HOBT: Hydroxybenzotriazole, NMM: *N*-methylmorpholine, XTT: (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide)

REFERENCES

(1) Roche, V. F. Cancer and chemotherapy. In Foye's principles of medicinal chemistry, 7th ed.; Lemke, T. L., Williams, D. A, Roche, V. F., William Zito, S., Lippincott Williams & Wilkins, Walter Kluwer Health, Baltimore and Philadelphia, **2013**; pp 1199-1266.

(2) American Cancer Society. Cancer Facts & Figures 2015. Atlanta: American Cancer Society; **2015**.

(3) Dunn, F. B. National Cancer Act: leaders reflect on 30 years of progress. *J. Natl. Cancer Inst.* **2002**, *94*, 8-9.

(4) Murciano-Goroff, Y. R. Philanthropic partnerships and the future of cancer research. *Nat. Rev. Cancer.* **2015**, *15*, 125-129.

(5) Gilchrest, B. A.; Eller, M. S. Cancer therapeutics: smart and smarter. *Drugs Future.* **2009**, *34*, 205-216.

(6) Dobbelsiein, M.; Moll, U. Targeting tumour-supportive cellular machineries in anticancer drug development. *Nat. Rev. Drug Disc*. **2014**, *13*, 179-196.

(7) Puyo, S.; Montaudon, D.; Pourquier, P. From old alkylating agents to new minor groove binders. *Crit. Rev. Oncol. Hematol.* **2014**, *89*, 43-61.

(8) Longley, D. B.; Harkin, D. P.; Johnston, P. G. 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer.* **2003**, *3*, 330-338.

(9) Cerqueira, N. M. F. S. A; Fernandes, P. A.; Ramos, M. J. Understanding ribonucleotide reductase inactivation by Gemcitabine. *Chem. Eur. J.* **2007**, *13*, 8507-8515.

(10) Bailly, C. Contemporary challenges in the design of topoisomerase II inhibitors for cancer chemotherapy. *Chem. Rev*. **2012**, *112*, 3611-3640.

(11) Ali, A.; Bhattacharya, S. DNA binders in clinical trials and chemotherapy. *Bioorg. Med. Chem.* **2014**, *22*, 4506-4521.

(12) Cui, J. J. A new challenging and promising era of tyrosine kinase inhibitors. *ACS Med. Chem. Lett*. **2014**, *5*, 272-274.

(13) Steeghs. N.; Nortier, J. W. R.; Gelderblom, H. Small molecule tyrosine kinase inhibitors in the treatment of solid tumors: an update of recent developments. *Ann. Surg. Oncol*. **2007**, *14*, 942-953.

(14) Zheng, Y. G; Wu, J.; Chen, Z.; Goodman, M. Chemical regulation of epigenetic modifications: opportunities for new cancer therapy. *Med. Res. Rev.* **2008**, *28*, 645-687.

(15) Bhat, R.; Tummalapalli, S. R.; Rotella, D. P. Progress in the discovery and development of heat shock protein 90 (Hsp90) inhibitors. *J. Med. Chem.* **2014**, *57*, 8718-8728.

(16) Huber, E. M.; Groll, M. Inhibitors for the immuno and constitutive proteasome: current and future trends in drug development. *Angew. Chem. Int. Ed*. **2012**, *51*, 8708-8720.

(17) Pinna, G. A.; Murineddu, G.; Murruzzu, C.; Zuco, V.; Zunino, F.; Cappelletti, G.; Artali, R.; Cignarella, G.; Solano, L.; Villa, S. Synthesis, modelling and antimitotic properties of tricyclic systems characterised by a 2-(5-phenyl-*1H*-pyrrol-3-yl)-1,3,4-oxadiazole moiety. *Chem. Med. Chem*. **2009**, *4*, 998-1009.

(18) Jones, P.; Wilcoxen, K.; Rowley, M.; Toniatti, C. Niraparib: a poly (ADP) polymerase (PARP) inhibitor for the treatment of tumors with defective homologous recombination. *J. Med. Chem*. **2015**, *58*, 3302-3314.

(19) Risinger, A. L.; Bennet, M. J.; Rohena, C. C.; Peng, J.; Schriemer, D. C.; Mooberry, S. L. Taccalonolide binding to tubulin imparts microtubule stability and potent in vivo activity. *Cancer Res.* **2013**, *73*, 6780-6792.

(20) Tinley, T. L.; Randall-Hlubek, D. A.; Leal, R. M.; Jackson, E. M.; Cessac, J. W.; Quada, J. C. Jr; Hemscheidt, T. K.; Mooberry, S. L. Taccalonolides E and A: plant-derived steroids with microtubule-stabilizing activity. *Cancer Res*. **2003**, *63*, 3211-3220.

(21) Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. A family of drug transporters: the multidrug resistance-associated proteins. *J. Natl. Cancer Inst*. **2000**, *92*,1295–1302.

(22) Hammond, C. L.; Marchan, R.; Krance, S. M.; Ballatoru, N. Glutathione export during apoptosis requires functional multidrug resistance associated proteins. *J. Biol. Chem*. **2007**, *282,* 14337-14347.

(23) Dean, M.; Rzhetsky, A.; Allikmets, R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res*. **2001**, *11*, 1156-1166.

(24) Ma, Y.; Wink, M. The beta-carboline alkaloid harmine inhibits BCRP and can reverse resistance to the anticancer drugs mitoxantrone and camptothecin in breast cancer cells. *Phytother Res*. **2010**, *24*, 146-149.

(25) Burkhart, C. A.; Watt, F.; Murray, J.; Pajic, M.; Prokvolit, A.; Xue, C.; Flemming, C.; Smith, J.; Purmal, A.; Isachemko, N.; Komarov, P. G.; Gurova, K.; Sartorelli, A. C.; Marshall, G. M.; Norris, M. D.; Gudkov, A. V.; Haber, M. Small-molecule multidrug resistance-associated protein 1 inhibitor reversan increases the therapeutic index of chemotherapy in mouse models of neuroblastoma. *Cancer Res.* **2009***, 69*, 6573–6580.

(26) Henderson, M. J.; Haber, M.; Porro, A.; Munoz, M. A.; Iraci, N.; Xue, C.; Murray, J.; Flemming, C. L.; Smith, J.; Fletcher, J. I.; Gherardi, S.; Kwek, C. K.; Russel, A. J.; Valli, E.; London, W. B.; Buxton, A. B.; Ashton, L. J.; Sartorelli, A. C.; Chon, S. L.; Schwab, M.; Marshall, G. M.; Perini, G.; Norris, M. D. ABCC multidrug transporters in childhood neuroblastoma: clinical and biological effects independent of cytotoxic drug efflux. *J. Natl. Cancer Inst.* **2011***, 103*, 1236–1251.

(27) Tivnan, A.; Zakaria, Z.; O'Leary, C.; Kögel, D.; Pokorny, J. L.; Sarkaria, J. N. and Prehn J. Inhibition of multidrug resistance protein 1 (MRP1) improves chemotherapy drug response in primary and recurrent glioblastoma multiforme. *Front. Neurosci.* **2015**, *9,* 218.

(28) Brown, J. M.; Attardi, L. D. The role of apoptosis in cancer development and treatment response. *Nat. Rev. Cancer.* **2005**, *5*, 231-237.

(29) Boyer, M. J.; Tannock, I. F. Cellular and molecular basis of chemotherapy**.** *In The Basic Science of Oncology* Edited by: Tannock IF, Hill RP. New York: McGraw-Hill. **1998**, 350-369.

(30) Hartwell, L. H.; Kastan, M. B. Cell cycle control and cancer. *Science*. **1994**, *266*, 1821–1828.

(31) McDonald, E. R. 3rd; El-Deiry, W.S. Cell cycle control as a basis for cancer drug development. *Int. J. Oncol*. **2000**, *16*, 871–886.

(32) Suganuma, M.; Kawabe, T.; Hori, H.; Funabiki, T.; Okamoto, T. Sensitization of cancer cells to DNA damage-induced cell death by specific cell cycle G2 checkpoint abrogation. *Cancer Res.* **1999**, *59*, 5887-5891.

(33) Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell.* **1997**, *88,* 323 – 331

(34) Jordan, M. A.; Wilson, L. Microtubules as a target for anticancer drugs. *Nat. Rev. Cancer*. **2004**, *4*, 253-265.

(35) Vousden, K. H.; Lu, X. Live or let die: the cell's response to p53. *Nat. Rev. Cancer*. **2002**, *2,* 594-604.

(36) Erol, A. Deciphering the intricate regulatory mechanisms for the cellular choice between cell repair, apoptosis or senescence in response to damaging signals. *Cell Signal*. **2011**, *23*, 1076–1081.

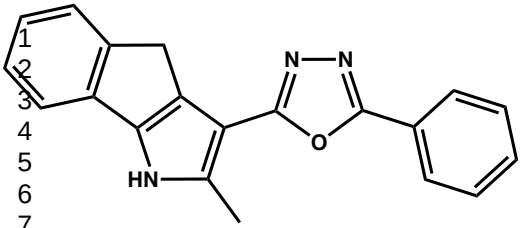
(37) Aylon, Y.; Oren, M. Living with p53, dying of p53. *Cell*, **2007**, *130,* 597–600.

(38) Innocente, S. A.; Abrahamson, J. L.; Cogswell, J. P.; Lee, J. M. p53 regulates a G2 checkpoint through cyclin B1. *Proc. Natl. Acad. Sci. USA* **1999**, *96,* 2147-2152.

(39) Badie, C.; Bourhis, J.; Sobczak-Thepot, J.; Haddada, H.; Chiron, M.; Janicot, Janot, F.; Tursz, M.; Vassal, G. p53-dependent G2 arrest associated with a decrease in cyclins A2 and B1 levels in a human carcinoma cell line. *Br. J. Cancer* **2000**, *82*, 642-650.

(40) Sandri, M. I.; Isaacs, R. J.; Ongkeko, W. M.; Harris, A. L.; Hickson, I. D.; Broggini, M.; Vikhanskaya F. p53 regulates the minimal promoter of the human topoisomerase IIalpha gene. *Nucleic Acids Res*. **1996**, *24*, 4464-4470.

(41) Anderson, H.; Roberge, M. Topoisomerase II inhibitors affect entry into mitosis and chromosome condensation in BHK cells. *Cell Growth Differ.* **1996***, 7*, 83-90.



2j

DMSO

2j