European Journal of Medicinal Chemistry 79 (2014) 382–390

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Substituted *E*-3-(3-indolylmethylene)1,3-dihydroindol-2-ones with antiproliferative activity. Study of the effects on HL-60 leukemia cells

Alberto Leoni [a], Alessandra Locatelli [a], Rita Morigi [a], Mirella Rambaldi [a,*], Concettina Cappadone [a], Giovanna Farruggia [a], Stefano Iotti [a], Lucia Merolle [a], Maddalena Zini [b], Claudio Stefanelli [c]

[a] Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy
[b] Department of Biomedical and Neuromotor Sciences, University of Bologna, 40126 Bologna, Italy
[c] Department for Life Quality Studies, University of Bologna, Rimini Campus, 47921 Rimini, Italy

ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form
1 April 2014
Accepted 2 April 2014
Available online 3 April 2014

Keywords:
Indolyl-indol-2-one
Knoevenagel reaction
Antiproliferative activity
Apoptosis
Divalent cations

ABSTRACT

The synthesis of new substituted *E*-3-(3-indolylmethylene)1,3-dihydroindol-2-ones is reported. The antiproliferative activity was evaluated according to protocols available at the National Cancer Institute (NCI), Bethesda, MD. The action of the most active compound **10** was further investigated in HL-60 leukemia cells. Results obtained show that it causes a block in cell cycle progression, with cell arrest in the G2/M phase, associated with activation of apoptosis accompanied with increased oxidative stress and deregulation of the homeostasis of divalent cations, with significant increase in the cellular concentrations of free Ca^{2+} and Mg^{2+}.

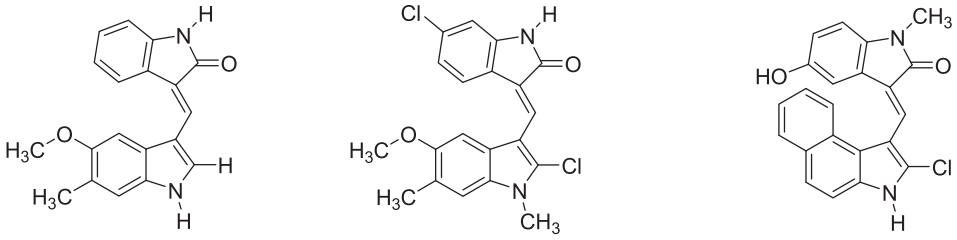
1. Introduction

Some of our previous studies were devoted to the synthesis of indolylmethylene-1,3-dihydroindol-2-one derivatives obtained through the Knoevenagel condensation [1–6]. This reaction allows to obtain adducts in a relatively easy way by reacting an aldehyde with an activated methylene group, thus permitting the generation of large series of compounds. Through this strategy, it was possible to identify several compounds endowed with a marked inhibition of cellular proliferation. The derivatives reported in Chart 1 were among the most potent obtained.

Abbreviations: NCI, National Cancer Institute; DTP, Developmental Therapeutics Program; GI, growth inhibition; TGI, total growth inhibition; LC, lethal concentration; BEC, Biological Evaluation Committee; DMSO, dimethyl sulfoxide; ROS, Reactive oxygen species; DCFDA, dichlorofluoresceine diacetate; DCF, dichlorofluorescein; PBS, phosphate buffered saline; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic; EGTA, ethylene glycol tetraacetic acid.

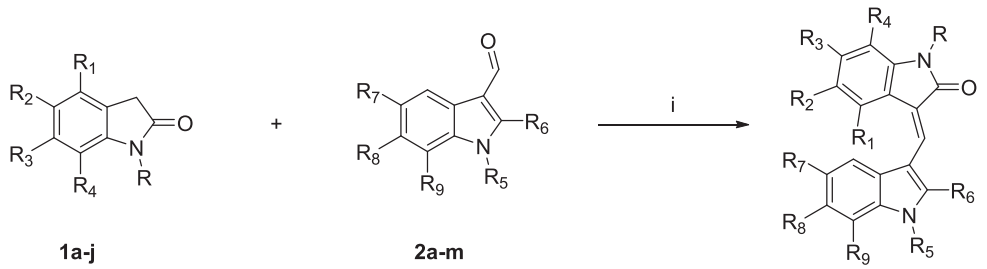
* Corresponding author.
E-mail address: mirella.rambaldi@unibo.it (M. Rambaldi).

http://dx.doi.org/10.1016/j.ejmech.2014.04.004
0223-5234/



GI_{50} 0.13 μM GI_{50} 0.03 μM GI_{50} 0.03 μM

Chart 1. Most active compounds reported in ref. **1**–**6**.



i = methanol, piperidine (for compounds **4**, **5**, **8**, **10**, **11**, **14-16**, **18-23**); acetic acid, 37% HCl (for compounds **3**, **6**, **7**, **9**, **12**, **13**, **17**); methanol, NH_4OH conc (for compounds **25-28**) and toluene, *p*-toluenesulfonic acid (for compound **24**)

Scheme 1. Synthesis of *E*-3-(3-indolylmethylene)1,3-dihydroindol-2-ones derivatives.

Table 1
New *E*-3-(3-indolylmethylene)1,3-dihydroindol-2-ones derivatives.

Comp	Starting compound **1**						Starting compound **2**					
		R	**R_1**	**R_2**	**R_3**	**R_4**		**R_5**	**R_6**	**R_7**	**R_8**	**R_9**
3	**a**	H	OCH_3	H	H	OCH_3	**a**	H	Cl	H	H	H
4	**a**	H	OCH_3	H	H	OCH_3	**b**	H	Cl	OCH_3	CH_3	H
5	**a**	H	OCH_3	H	H	OCH_3	**c**	Bn	Cl	OCH_3	CH_3	H
6	**a**	H	OCH_3	H	H	OCH_3	**d**	H	Cl	H	CBR	
7	**b**	H	OCH_3	OCH_3	OCH_3	H	**a**	H	Cl	H	H	H
8	**c**	H	H	H	H	H	**e**	Bn	Cl	H	H	H
9	**d**	H	H	OH	H	H	**f**	PhEt	Cl	H	H	H
10	**c**	H	H	H	H	H	**g**	4-MPhEt	Cl	H	H	H
11	**c**	H	H	H	H	H	**h**	Phprop	H	H	H	H
12	**c**	H	H	H	H	H	**i**	Phprop	Cl	H	H	H
13	**e**	H	H	Cl	H	H	**i**	Phprop	Cl	H	H	H
14	**f**	H	H	OCH_3	H	H	**i**	Phprop	Cl	H	H	H
15	**d**	H	H	OH	H	H	**i**	Phprop	Cl	H	H	H
16	**c**	H	H	H	H	H	**j**	Morphet	Cl	H	H	H
17	**f**	H	H	OCH_3	H	H	**k**	cinn	Cl	H	H	H
18	**f**	H	H	OCH_3	H	H	**l**	3,4,5-TM-Bn	Cl	OCH_3	CH_3	H
19	**d**	H	H	OH	H	H	**l**	3,4,5-TM-Bn	Cl	OCH_3	CH_3	H
20	**g**	H	H	OCH_3	CH_3	H	**l**	3,4,5-TM-Bn	Cl	OCH_3	CH_3	H
21	**e**	H	H	Cl	H	H	**l**	3,4,5-TM-Bn	Cl	OCH_3	CH_3	H
22	**h**	H	Cl	H	H	H	**l**	3,4,5-TM-Bn	Cl	OCH_3	CH_3	H
23	**i**	Bn	H	H	H	H	**a**	H	Cl	H	H	H
24	**c**	H	H	H	H	H	**m**	Ph	OCH_3	H	H	H
25	**e**	H	H	Cl	H	H	**m**	Ph	OCH_3	H	H	H
26	**f**	H	H	OCH_3	H	H	**m**	Ph	OCH_3	H	H	H
27	**j**	CH_3	H	Cl	H	H	**m**	Ph	OCH_3	H	H	H
28	**a**	H	OCH_3	H	H	OCH_3	**m**	Ph	OCH_3	H	H	H

Bn = benzyl; CBR = condensed benzene ring; PhEt = phenylethyl; 4-MPhEt = 4-methoxyphenylethyl; Phprop = phenylpropyl; Morphet = morfolinethyl; cinn = cinnamic; 3,4,5-TM-Bn = 3,4,5-trimethoxybenzyl; Ph = phenyl.

In this paper we wish to continue the study of the antiproliferative activity and of the mechanism of action of new analogues bearing the indolylmethylene-1,3-dihydroindol-2-one scaffold (Scheme 1 and Table 1) in which the substituents of the two heterocyclic systems were chosen as described below.

Considering the positive effect of the methoxy groups at the chloroindole portion previously demonstrated, we have proceeded to the synthesis of compounds **3**–**7** in which the introduction of methoxy groups at the indolinone nucleus has been considered, in order to study the effect of this shift.

Table 2
Nine subpanels at five concentrations: growth inhibition, cytostatic and cytotoxic activity (μM) of the selected compounds.

N. NSC	Comp[a]	Modes	Leukemia	NSCLC	Colon	CNS	Melanoma	Ovarian	Renal	Prostate	Breast	MG-MID[b]
746082	**3**[c]	GI_{50}	1.51	3.55	2.24	2.75	3.39	2.51	3.39	3.47	1.62	2.57
		TGI	56.23	22.39	8.91	13.49	16.22	15.14	16.22	19.95	12.59	16.60
		LC_{50}	–	74.13	33.88	44.67	46.77	41.69	53.70	51.29	64.57	54.95
745261	**7**[c]	GI_{50}	0.55	1.86	1.29	1.58	1.86	1.51	1.35	1.17	1.62	1.41
		TGI	7.08	9.12	4.27	6.31	6.61	9.77	5.37	5.50	8.13	6.92
		LC_{50}	42.66	67.61	18.20	35.48	30.90	77.62	36.31	–	66.07	44.67
744492	**8**	GI_{50}	3.63	14.79	19.95	16.60	8.91	20.89	20.42	20.42	12.88	13.49
		TGI	21.38	57.54	60.26	47.86	32.36	66.07	70.79	54.95	64.57	50.12
		LC_{50}	75.86	–	95.50	93.33	87.10	95.50	–	–	–	93.33
751251	**9**	GI_{50}	3.39	7.08	9.77	10.47	7.08	9.12	9.33	6.76	3.98	7.24
		TGI	77.62	50.12	33.88	52.48	28.18	64.57	54.95	47.86	25.12	44.67
		LC_{50}	–	–	72.44	95.50	74.13	95.50	95.50	–	–	91.20
750019	**10**[c]	GI_{50}	0.35	0.43	0.28	0.34	0.46	0.34	0.48	0.35	0.32	0.38
		TGI	15.49	15.14	1.82	8.32	9.12	13.49	18.62	19.50	6.31	9.77
		LC_{50}	–	74.13	52.48	58.88	63.10	69.18	72.44	54.95	–	72.44
743939	**12**[c]	GI_{50}	8.91	14.13	12.02	16.60	13.80	12.02	13.80	19.05	10.00	12.88
		TGI	50.12	72.44	60.26	75.86	67.61	72.44	67.61	–	72.44	67.61
751960	**13**[c]	GI_{50}	2.88	2.24	1.95	2.82	2.04	2.45	1.86	2.00	2.19	2.24
		TGI	81.28	8.91	5.62	10.23	5.75	23.44	9.77	6.46	9.12	11.48
		LC_{50}	–	28.84	19.05	50.12	35.48	60.26	17.78	34.67	42.66	36.31
751963	**17**	GI_{50}	2.00	2.24	1.32	2.04	2.40	2.09	1.91	2.09	1.86	2.00
		TGI	–	37.15	21.88	–	38.90	41.69	30.20	16.22	36.31	38.90
742198	**18**	GI_{50}	6.17	20.42	10.96	16.22	14.79	33.11	29.51	14.45	10.23	15.85
		TGI	77.62	97.72	85.11	75.86	75.86	–	–	95.50	57.54	83.18
741774	**19**[c]	GI_{50}	1.41	2.40	2.29	2.04	1.45	2.34	2.75	2.29	1.41	2.00
		TGI	18.62	32.36	48.98	34.67	11.48	43.65	33.11	50.12	34.67	29.51
742490	**20**	GI_{50}	3.24	10.96	16.60	48.98	25.12	30.90	38.90	5.50	10.00	16.60
		TGI	42.66	66.07	–	–	–	–	–	87.10	–	85.11
742489	**21**	GI_{50}	1.86	2.29	2.19	2.34	2.29	3.72	2.95	1.78	2.09	2.40
		TGI	4.79	5.89	5.62	6.03	5.75	13.80	7.24	3.89	5.50	6.31
		LC_{50}	15.85	16.22	29.51	23.44	17.78	39.81	18.62	8.91	22.39	20.89
742488	**22**	GI_{50}	2.45	3.24	2.88	3.02	2.88	3.98	3.24	1.91	2.95	3.02
		TGI	14.13	12.02	10.00	12.59	8.91	22.91	11.75	5.13	15.14	12.30
		LC_{50}	51.29	47.86	51.29	40.74	51.29	66.07	45.71	17.38	61.66	50.12
743995	**23**	GI_{50}	2.95	6.61	4.17	4.90	5.13	9.12	5.25	6.17	4.17	5.01
		TGI	21.38	85.11	79.43	69.18	61.66	79.43	77.62	–	56.23	64.57
742498	**24**[c]	GI_{50}	0.52	2.14	1.05	2.19	1.48	1.38	1.12	1.12	1.23	1.32
		TGI	2.63	8.32	3.24	8.51	3.98	4.07	4.07	3.47	4.47	4.57
		LC_{50}	50.12	27.54	7.94	30.90	10.47	15.85	13.18	19.05	27.54	19.05
747146	**25**	GI_{50}	2.34	9.55	6.31	5.25	5.62	8.51	8.51	9.33	4.27	5.89
		TGI	8.91	24.55	25.70	19.05	18.20	23.99	21.88	23.99	13.80	18.62
		LC_{50}	58.88	57.54	60.26	52.48	51.29	66.07	51.29	60.26	52.48	56.23
747433	**26**[c]	GI_{50}	0.76	2.51	1.74	0.98	2.40	2.00	2.29	2.63	0.63	1.58
		TGI	4.47	17.38	10.96	9.55	16.98	17.38	17.38	13.18	7.24	12.02
		LC_{50}	58.88	63.10	42.66	38.90	50.12	66.07	63.10	52.48	47.86	53.70
Vincristine sulfate[d]		GI_{50}	0.10	0.25	0.10	0.13	0.16	0.32	0.32	0.13	0.32	0.20
		TGI	15.85	15.85	3.98	6.31	7.94	19.95	19.95	6.31	7.94	10.00
		LC_{50}	630.96	251.19	79.43	199.53	251.19	316.23	251.19	316.23	316.23	251.19

[a] Highest conc. = 10^{-4} M unless otherwise reported; only value >100 μM are reported. The compound exposure time was 48 h.
[b] Mean Graph MIDpoint i.e. the calculated panel mean.
[c] Mean of two separate experiments.
[d] Highest conc. = 10^{-3} M.

We have also focused the study of new substituents at the nitrogen (R_5) of the chloroindole moiety. Until now, we have considered methyl, phenyl, benzyl and substituted benzyl group. In particular, good results were obtained with benzyl and *p*-chlorobenzyl which led to compounds showing GI_{50} 0.65 and 0.36 μM respectively [4]. The synthesis of compounds **8**–**15** enables us to study the effect of the increment of the distance between the nitrogen and the phenyl whereas in compound **16** the phenyl has been substituted by the morpholine and in compound **17** the effect of the introduction of a double bond in the propylphenyl group has been considered. Moreover, since the trimethoxyphenyl group is contained in several antitumor agents (such as colchicine, combretastatin, podophyllotoxin) we planned the synthesis of compounds **18**–**22** bearing the trimethoxybenzyl group at the chloroindole nitrogen (R_5). Furthermore, the effect of the shift of the benzyl group from the nitrogen of the chloroindole to that of the indolinone (compound **23**) has been considered.

Finally, we have pursued the study of the substituents at the 2 position of the indole portion (R_6). In our previous studies this position was substituted with a chlorine or unsubstituted. Through the synthesis of compounds **24**–**28** the effect of a methoxy group at this position has been investigated.

The cytotoxic activities of all new compounds were evaluated according to Developmental Therapeutics Program (DTP), National Cancer Institute (NCI), Bethesda, MD, drug screen protocols. For some insights into the biological effects of these derivatives, the most active compound **10** was submitted to additional studies concerning the effect on cell cycle progression and on induction of apoptosis.

2. Chemistry

Most of the compounds were prepared by means of the single step Knoevenagel reaction between the indolinones **1** and the aldehydes **2** in methanol/piperidine. For compounds **3**, **6**, **7**, **9**, **12**, **13** and **17** a mixture of acetic acid/hydrochloric acid has been employed, for the reaction of compounds **25**–**28** has been used methanol and NH_4OH conc, while for compound **24** has been used toluene and *p*-toluenesulfonic acid.

Compounds were obtained as almost pure geometrical isomers which, according to the usual NOE experiments described in the previous papers [7,8] were assigned to the E configuration. The oxindoles **1c** and **1e** are commercially available whereas the other starting compounds (oxindoles and aldehydes) have been prepared according to the literature [4,6,9–20], except the aldehydes **2g, 2j**–**l** reported under the experimental section.

3. Biology

3.1. Cell-based assays

In a preliminary test, compounds were assayed at a single high concentration (10^{-5} M) in the full NCI 60 cell panel. This panel is organized into subpanels representing leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and central nervous system. Only compounds that satisfy predetermined threshold inhibition criteria in a minimum number of cell lines progress to the full five-concentration assay. The threshold inhibition criteria for progression to the five-concentration screen was selected to efficiently capture compounds with antiproliferative activity based on the analysis of historical DTP screening data. The result is expressed as the percent growth of treated cells at the test concentration of 10^{-5} M following 48 h of incubation (unpublished results).

Compounds **4**–**6**, **11**, **14**–**16** and **27**–**28** were not considered active enough to enter the five-concentration test, whereas all the others were subjected to this screen. The compounds were dissolved in dimethyl sulfoxide (DMSO) and evaluated using five concentrations at 10-fold dilutions (the highest being 10^{-4} M) following 48 h of incubation.

Table 2 reports the results obtained (vincristine is reported for comparison purposes), expressed as μM at three assay end points: the 50% growth inhibitory power (GI_{50}), the cytostatic effect (TGI = total growth inhibition), and the cytotoxic effect (LC_{50}). For some compounds the five-concentrations test was repeated and no significant differences were found. For these compounds the data reported in Table 2 are the mean values of the two experiments.

The tested compounds showed a mean GI_{50} between 0.38 and 16.60 μM, and compounds **3** and **26** were submitted to BEC (Biological Evaluation Committee) for possible future development.

In light of the NCI 60 results, the following considerations may be done.

The introduction of methoxy groups at the oxindole portion did not lead to an improvement of activity, in fact among the five compounds synthesized only two (**3**, **7**) were tested also in the five concentrations assay, although compound **3** was selected by BEC for the great difference between GI_{50} and LC_{50}, and compound **7** showed selectivity towards leukemia cell lines (mean GI_{50} 0.55 μM).

Interesting results have been obtained studying the effect of the substituents at the nitrogen of the chloroindole (R_5). Considering compounds **8**, **10** and **12**, which are different only for R_5, it can be noted that the benzyl and phenylpropyl groups determine about the same activity: compound **8** and **12** showed mean GI_{50} of 13.49 and 12.88 μM, respectively. A notable increment of activity was obtained when R_5 is a 4-methoxyphenylethyl group, in fact compound **10** showed mean GI_{50} of 0.38 μM therefore proving to be the most active of the series. Comparing compounds **9** and **15**, it is evident that the substitution of the phenylethyl with a phenylpropyl group was detrimental for the activity in fact compound **15** did not progress to the full five-concentration assay whereas compound **9** showed mean GI_{50} 7.24 μM. However, in the presence of the phenylpropyl group, the activity seems to be strongly influenced by the substituent at the indolinone moiety (R_2): compounds **14** (R_2 = OCH_3) and **15** (R_2 = OH) did not enter the five-concentration assay, whereas compound **13** (R_2 = Cl) showed mean GI_{50} of 2.24 μM. The introduction of the morpholinethyl group was disadvantageous and led to an inactive compound (**16**). Good results were achieved by introducing the cinnamoyl group (compound **17** showed mean GI_{50} of 2.00 μM), as well as the 3,4,5-trimethoxybenzyl group (**18**–**22**): all the derivatives progressed to the full five-concentration assay and three of them (**19**, **21** and **22**) showed a mean GI_{50} between 2.00 and 3.00 μM.

Shifting of the N-benzyl from the chloroindole (**8**) to the oxindole nitrogen (**23**) led to a significant difference in the biological behavior: mean GI_{50} of 13.49 μM, vs 5.01 μM.

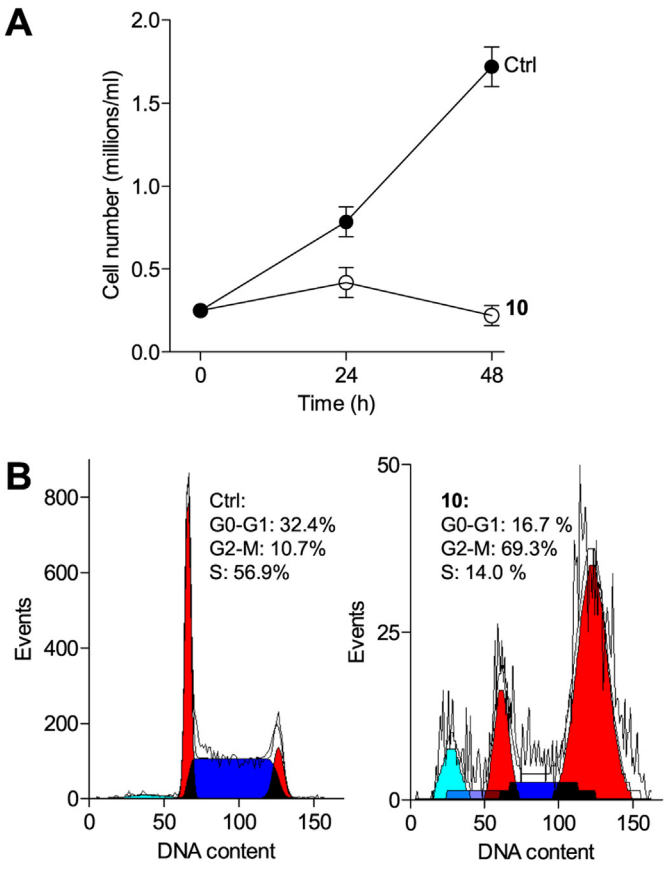


Fig. 1. Effects of compound **10** on the growth of HL-60 cells. (**A**) Rate of cell growth determined as total cell number. Control cells and cells treated with compound **10** (1 μM) were incubated and viable cells were counted daily. Means ± s.e.m. of three determinations. (**B**) Effect of compound **10** on cell cycle. Cells were incubated for 24 h with the vehicle (Ctrl) or with compound **10** (1 μM), afterward cell cycle distribution was determined by flow cytometry. Panels report the cytofluorimeter outputs obtained in one typical experiment repeated twice with similar results. The percentages of cells in the different phases of the cell cycle (G0-G1; G2-M; S) are reported. Following treatment with **10**, most cells are in the G2-M phase and a well detectable fraction of DNA is present as a pre-G1 peak (light blue peak). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

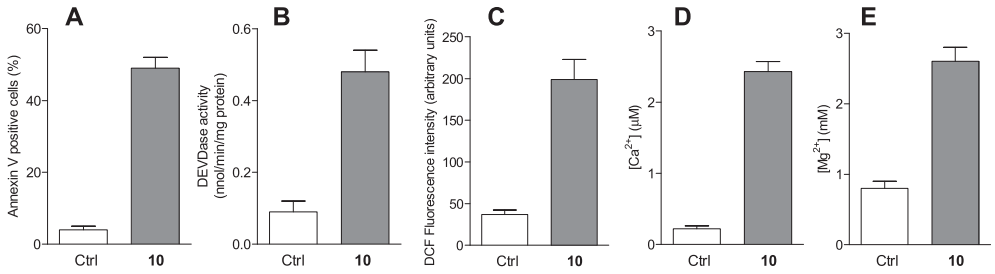


Fig. 2. Effect of compound **10** on biochemical parameters in HL-60 cells. Control cells and cells treated with 1 μM compound **10** were incubated for 24 h, afterward the following parameters were measured. (**A**) The exposure of phosphatidylserine in the outer side of the plasma membrane was determined by flow cytometry after labelling with Annexin V/FLUOS. (**B**) Caspase activity acting on the peptide sequence DEVD (DEVDase activity) was measured in cellular extracts. (**C**) Detection of oxidative stress by flow cytometry after DCFDA staining. The increase in the fluorescence intensity (DCF-positive cells) in cells incubated in the presence of **10**, indicates an increase in intracellular peroxides. (**D**) Intracellular content of Ca^{2+} was assayed by the INDO-1/AM probe. (**E**) Intracellular content of Mg^{2+} measured by the Mag-Fura2 probe. Results depicted in each panel are means ± s.e.m. of three determinations.

Although the evaluation of the antitumor activity of the compounds lacking chlorine at the 2-position (R_6) of the indole portion needs to be further investigated, the introduction at this position of a methoxy group gave promising results (**24**–**26**), in particular compound **24**, which showed mean GI_{50} of 1.32 μM and demonstrated to be effective mainly versus leukemia cells (mean GI_{50} 0.52 μM).

3.2. Effect on growth and death of HL-60 cells

To give some insights into the biological effects of the new derivatives, the most active compound **10** was submitted to additional studies using HL-60 leukemia cells. Fig. 1A shows that compound **10** at 1 μM concentration strongly reduced cell proliferation within 24 h of treatment. After 48 h, the cell number was decreased, indicating the onset of cytotoxicity. In order to assess whether the antiproliferative effect of **10** was associated with interference with cell cycle progression, DNA profiles of cultured cells were examined by flow cytometry. Fig. 1B shows that the treatment with **10** caused a marked accumulation of HL-60 cells in the G2/M phase (69.3%), whereas only 10.7% of control cells were in the G2/M phase. In **10**-treated cells, the block in the G2/M phase of the cell cycle was associated with a well distinguishable pre-G1 peak in DNA, suggestive of DNA fragmentation, characteristic of apoptosis.

To confirm the activation of the apoptotic program, we determined the exposure of phosphatidylserine from the inner to the outer side of the plasma membrane, which is an early marker of apoptosis [21], utilizing the Annexin V-FLUOS staining method. Annexin V is a protein with high affinity for phospholipids, and cells undergoing apoptosis are marked from early stages with Annexin V-FLUOS, which binds to externalized phosphatidylserine. As shown in Fig. 2A, after 24 h of incubation, only 4% of control HL-60 cells were Annexin V positive, whereas this percentage rose to about 50% in cells incubated in the presence of compound **10**. To further confirm the onset of apoptosis, the activity of caspase proteases was assayed. In cells treated for 24 h with **10**, the activity of effector caspases cutting their substrate at level of the aminoacid motif DEVD (Asp-Glu-Val-Asp) was significantly increased (Fig. 2B). Recently, it has become evident that activation of autophagy is an important mechanism in the antiproliferative effect of anticancer molecules [22], however we did not observe any effect of **10** on autophagy in HL-60 cells, as detected by acridine orange staining or by LC3 protein processing, that represents a hallmark of autophagy [23] (data not shown).

In order to further characterize the biological effects of compound **10**, we next examined a few other biochemical parameters. Reactive oxygen species (ROS) and Ca^{2+} often have a critical role in the modulation of cell death because a wide variety of toxic molecules are known to affect cellular redox balance or Ca^{2+} concentration, that can both deeply influence cellular signaling [24]. To assess the production of ROS, we evaluated the intracellular oxidative stress by the probe dichlorofluoresceine diacetate (DCFDA), which is oxidized to the highly fluorescent dichlorofluorescein (DCF) by cellular peroxides. The treatment of HL-60 cells with compound **10** caused a well evident increase in DCF fluorescence indicating increased peroxide production (Fig. 2C). As regards Ca^{2+}, the concentration of free intracellular calcium ion, measured by the probe INDO-1/AM, was significantly increased in **10**-treated cells (Fig. 2D). Lastly, we wanted to determine whether even Mg^{2+} concentration was affected by **10**. In fact, recent work has shown that magnesium can be involved in both cell growth and apoptosis in cancer cells even though its role in these processes is not yet fully defined [25]. Magnesium can lead to activation of several enzymes, including nucleases, and exposure to elevated Mg^{2+} causes cell death [26]. To date, a very few studies have examined the effect of anticancer compounds on cellular magnesium. Fig. 2E shows that the exposure of HL-60 cells to **10**, causes the increase in the intracellular concentration of free Mg^{2+}, determined by the fluorescent probe Mag-Fura2.

Altogether, the presented data suggest a picture in which **10** causes a block in cell cycle progression, with cell arrest in the G2/M phase associated with increased oxidative stress and to a deregulation of the homeostasis of divalent cations Ca^{2+} and Mg^{2+}.

4. Conclusions

The synthesis of new *E*-3-(3-indolylmethylene)1,3-dihydroindol-2-ones led to compounds showing in the NCI-60 cell test mean GI_{50} values in the micromolar or sub-micromolar range. Two compounds (**3** and **26**) demonstrated a particularly interesting activity profile and were selected by BEC for possible future development.

The introduction at the chloroindole nitrogen of a 4-methoxyphenylethyl group allowed to obtain compound **10**, which showed submicromolar mean GI_{50} values in all the nine NCI subpanels, thus revealing to be the most active compound of the series.

Compound **10** was also submitted to additional studies in HL-60 leukemia cells which showed that it exerts the antitumor activity by inhibition of cell proliferation and activation of apoptosis. Compound **10** causes a block in cell cycle progression, with arrest in the G2/M phase, associated with increased intracellular formation of peroxides and deregulation of the homeostasis of divalent cations, with significant increase in the cellular content of free Ca^{2+} and Mg^{2+}. These biochemical events probably represent the triggers for the induction of apoptosis that leads to cancer cell death.

Considering all these findings and that compound **10** is the first indolylmethyleneindol-2-one bearing a 4-methoxyphenylethyl

group we have synthesized, it can be considered as a lead which deserves to generate new analogues.

5. Experimental section

5.1. Chemistry

All the compounds prepared have a purity of at least 95% as determined by combustion analysis. The melting points are uncorrected. TLC was performed on Bakerflex plates (Silica gel IB2-F) and column chromatography on Kieselgel 60 (Merck): the eluent was a mixture of petroleum ether/acetone in various proportions. The IR spectra were recorded in nujol on a Nicolet Avatar 320 E.S.P.; ν_{max} is expressed in cm^{-1}. The ^1H NMR spectra were recorded in $(CD_3)_2SO$ on a Varian MR 400 MHz (ATB PFG probe); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz (abbreviations: ph = phenyl, bzind = benzoindole, ind = indole, ox = oxindole). The oxindoles **1c** and **1e** are commercially available. The following compounds were prepared according to the literature: **1a** [9], **1b** [10], **1d** [11], **1f** [12], **1g** [13], **1h** [14], **1i** [15], **1j** [16], **2a** [17], **2c** [4], **2d** [6], **2e** [18], **2f**, **h**–**i** [19], **2m** [20].

5.1.1. General procedure for the preparation of the new aldehydes (**2g**, **2j**–**l**)

The 2-chloro-1*H*-indole-3-carbaldehyde or 2-chloro-5-methoxy-6-methyl-1*H*-indole-3-carbaldehyde (10 mmol) was dissolved in DMF (10 mL) and treated, under stirring, with NaH (15 mmol). The mixture was stirred at room temperature for 10 min and it was added with 4-methoxyphenethyl bromide, 2-morpholinoethyl chloride, 3-bromo-1-phenyl-1-propene or 3,4,5-trimethoxybenzyl chloride (12 mmol) respectively. The reaction mixture was maintained at 100 °C for 3–5 h (according to TLC test), after cooling, was poured into ice water. The expected compounds were collected by filtration and crystallized from ethanol.

*5.1.1.1. 2-Chloro-1-[2-(4-methoxyphenyl)ethyl]-1H-indole-3-carbaldehyde (**2g**).* Yield 77%, mp 85–90 °C. IR: 1650, 1504, 1239, 1033, 744. ^1H NMR (DMSO-d_6): 2.99 (2H, *t*, CH_2, $J = 7.4$), 3.69 (3H, s, OCH_3), 4.50 (2H, *t*, CH_2, $J = 7.4$), 6.79 (2H, d, ph, $J = 8.7$), 7.03 (2H, d, ph, $J = 8.7$), 7.32 (2H, m, ind), 7.65 (1H, m, ind), 8.09 (1H, m, ind), 9.95 (1H, s, CHO). Anal. Calcd for $C_{18}H_{16}ClNO_2$ (MW 313.78): C, 68.90; H, 5.14; N 4.46. Found: C, 69.04; H, 5.02; N, 4.78.

*5.1.1.2. 2-Chloro-1-(2-morpholin-4-ylethyl]-1H-indole-3-carbaldehyde (**2j**).* Yield 45%, mp 88–92 °C. IR: 1605, 1500, 1110, 1045, 755. ^1H NMR (DMSO-d_6): 3.72 (4H, m, CH_2), 3.77 (2H, *t*, CH_2, $J = 5.0$), 3.89 (2H, m, CH_2), 4.18 (4H, m, CH_2), 6.99 (2H, m, ind), 7.04 (1H, m, ind), 7.85 (1H, s, ind), 9.82 (1H, s, CHO). Anal. Calcd for $C_{15}H_{17}ClN_2O_2$ (MW 292.76): C, 61.54; H, 5.85; N, 9.57. Found: C, 61.23; H, 5.73; N, 9.77.

*5.1.1.3. 2-Chloro-1-[(2E)-3-phenylprop-2-en-1-yl]-1H-indole-3-carbaldehyde (**2k**).* Yield 40%, mp 89–93 °C. IR: 1654, 1508, 1038, 970, 743. ^1H NMR (DMSO-d_6): 5.14 (2H, d, CH_2, $J = 5.4$), 6.43 (1H, dt, CH, $J = 16$, $J = 5.4$), 6.54 (1H, d, CH, $J = 16$), 7.24 (1H, *t*, ph, $J = 6.8$), 7.32 (4H, m, ph + ind), 7.40 (1H, *t*, ind, $J = 7.6$), 7.41 (1H, d, ind, $J = 7.6$), 7.73 (1H, d, ind, $J = 7.6$), 8.13 (1H, d, ph, $J = 6.8$), 10.06 (1H, s, CHO). Anal. Calcd for $C_{18}H_{14}ClNO$ (MW 295.76): C, 73.10; H, 4.77; N, 4.74. Found: C, 72.98; H, 4.93; N, 4.65.

*5.1.1.4. 2-Chloro-5-methoxy-6-methyl-1-(3,4,5-trimethoxybenzyl)-1H-indole-3-carbaldehyde (**2l**).* Yield 40%, mp: 157–158 °C. IR: 1651, 1588, 1129, 1045. ^1H NMR (DMSO-d_6): 2.25 (3H, s, CH_3), 3.61 (3H, s, OCH_3), 3.68 (6H, s, 2 × OCH_3), 3.83 (3H, s, OCH_3), 5.45 (2H, s, CH_2), 6.54 (2H, s, ph), 7.54 (1H, s, ind), 7.58 (1H, s, ind), 10.01 (1H, s, CHO). Anal. Calcd for $C_{21}H_{22}ClNO_5$ (MW 403.86): C, 62.45; H, 5.49; N, 3.47. Found: C, 62.47; H, 5.45; N, 3.45.

5.1.2. General procedure for the synthesis of compounds **4**, **5**, **8**, **10**, **11**, **14**–**16**, **18**–**23**, **25**–**28**

The appropriate compound **1** (10 mmol) was dissolved in methanol (100 mL) and treated with the equivalent of the appropriate aldehyde **2** and piperidine (1 mL). The reaction mixture was refluxed for 3–5 h (progress of the reaction followed by TLC), and the precipitate that formed on cooling was collected by filtration.

Compounds **16**, **25** and **27** were purified by column chromatography, compounds **16** and **25** with petroleum ether/acetone, and compound **27** with methylene chloride/acetone as the eluent. All the crude products were crystallized from ethanol except compound **4** ($CHCl_3$/MeOH) and compound **5** (acetone/petroleum ether).

For compounds **25**–**28** the yield was much lower, and an improvement was obtained by replacing piperidine with NH_4OH conc.

4. Yield 65%, mp > 320 °C. I.R.: 1672, 1573, 1263, 1030, 722. ^1H NMR: 2.24 (3H, s, CH_3), 3.74 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 6.58 (1H, d, ox, $J = 9.0$), 6.81 (1H, s, ind), 6.85 (1H, d, ox, $J = 9.0$), 7.12 (1H, s, ind), 8.02 (1H, s, CH), 10.48 (1H, s, NH), 12.28 (1H, broad, NH). Anal. Calcd for $C_{21}H_{19}ClN_2O_3$ (MW 382.84): C, 65.88; H, 5.00 N, 7.32. Found: C, 65.69; H, 4.87; N, 7.06.

5. Yield 62%, mp 247–250 °C. I.R.: 1693, 1594, 1259, 1109, 721. ^1H NMR: 2.22 (3H, s, CH_3), 3.74 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 6.52(2H, s, CH_2), 6.58 (1H, d, ox, $J = 9.0$), 6.85 (1H, s, ind), 6.87 (1H, d, ox, $J = 9.0$), 7.14 (2H, m, ph), 7.32 (4H, m, ph + ind), 8.03 (1H, s, CH), 10.51 (1H, s, NH). Anal. Calcd for $C_{28}H_{25}ClN_2O_4$ (MW 488.96): C, 68.78; H, 5.15 N, 5.73. Found: C, 68.58; H, 4.98; N, 5.48.

8. Yield 60%, mp 138–140 dec °C. I.R.: 3585, 3170, 1696, 1609, 743. ^1H NMR: 5.64 (2H, s, CH_2), 6.75 (2H, d, ph, $J = 4.2$), 6.88 (2H, d, $J = 8$), 7.26 (8H, m), 7.66 (1H, s, CH), 7.70 (1H, d, $J = 8$), 10.63 (1H, s, NH). Anal. Calcd for $C_{24}H_{17}ClN_2O$ (MW 384.86): C, 74.90; H, 4.45 N, 7.28. Found: C, 74.88; H, 4.48; N, 7.30.

10. Yield 85%, mp 161–168 °C. I.R.: 3125, 1698, 1602, 1245, 741. ^1H NMR: 3.03 (2H, *t*, CH_2, $J = 7$), 3.67 (3H, s, OCH_3), 4.54 (2H, *t*, CH_2, $J = 7.0$), 6.64 (1H, d, ox, $J = 7.4$), 6.76 (2H, d, ph, $J = 8.4$), 6.77 (1H, m, ox), 6.87 (1H, d, ox, $J = 7.4$), 7.03 (2H, d, ph, $J = 8.4$), 7.18 (3H, m, ind), 7.30 (1H, m, ind), 7.58 (1H, s, CH), 7.67 (1H, d, ind, $J = 8.2$), 10.60 (1H, s, NH). Anal. Calcd for $C_{26}H_{21}ClN_2O_2$ (MW 428.91): C, 72.81; H, 4.94; N, 6.53. Found: C, 72.46; H, 4.53; N, 6.49.

11. Yield 90%, mp 205–208 °C. I.R.: 3196, 1648, 1509, 1181, 736. ^1H NMR: 2.14 (2H, qui, CH_2, $J = 7.6$), 2.65 (2H, *t*, CH_2, $J = 7.6$), 4.33 (2H, *t*, CH_2, $J = 7.6$), 6.85 (1H, d, ox, $J = 7.2$), 6.99 (1H, *t*, ox, $J = 7.2$), 7.22 (8H, m), 7.58 (1H, m), 7.89 (1H, d, $J = 7.2$), 8.13 (1H, s, CH), 8.21 (1H, m), 9.49 (1H, s, ind-2), 10.54 (1H, s, NH). Anal. Calcd for $C_{26}H_{22}N_2O$ (MW 378.47): C, 82.51; H, 5.86; N, 7.40. Found: C, 82.37; H, 5.78; N, 7.27.

14. Yield 95%, mp 161–165 °C. I.R.: 3178, 1696, 1608, 1193, 743. ^1H NMR: 2.08 (2H, qui, CH_2, $J = 7.4$), 2.68 (2H, *t*, CH_2, $J = 7.4$), 3.35 (3H, s, OCH_3), 4.40 (2H, *t*, CH_2, $J = 7.5$), 6.36 (1H, s, ox-4), 6.77 (2H, m, ox-6,7), 7.26 (8H, m, ind + ph), 7.63 (1H, s, CH), 7.66 (1H, m, ind), 10.43 (1H, s, NH). Anal. Calcd for $C_{27}H_{23}ClN_2O_2$ (MW 442.94): C, 73.21; H, 5.23; N, 6.32. Found: C, 73.18; H, 5.36; N, 6.29.

15. Yield 68%, mp 188 dec °C. I.R.: 3240, 1686, 1608, 1189, 738. ^1H NMR: 2.08 (2H, qui, CH_2, $J = 7.6$), 2.70 (2H, *t*, CH_2, $J = 7.6$), 4.38 (2H, *t*, CH_2, $J = 7.6$), 6.27 (1H, d, ox-4, $J = 2.0$), 6.59 (1H, dd, ox-6, $J = 8.2$, $J = 2$), 6.67 (1H, d, ox-7, $J = 8.2$), 7.25 (8H, m, ind + ph), 7.56 (1H, s, CH), 7.64 (1H, d, ind, $J = 8.4$), 8.78 (1H, s, OH), 10.28 (1H, s, NH). Anal. Calcd for $C_{26}H_{21}ClN_2O_2$ (MW 428.91): C, 72.81; H, 4.94; N, 6.53. Found: C, 72.52; H, 4.87; N, 6.48.

16. Yield 35%, mp 210–214 °C. I.R.: 3119, 1685, 1565, 1206, 737. ^{1}H NMR: 3.43 (2H, *t*, CH_2, $J = 5.4$), 3.60 (4H, m, CH_2), 3.69 (2H, m, CH_2), 4.21 (4H, m, CH_2), 6.64 (1H, d, $J = 7.5$), 6.75 (1H, d, $J = 7.5$), 6.82 (1H, td, $J = 7.5$, $J = 1.2$), 6.87 (1H, d, $J = 7.5$), 6.96 (1H, td, $J = 7.5$, $J = 1.2$), 7.02 (1H, td, $J = 7.5$, $J = 1.2$), 7.07 (1H, td, $J = 7.5$, $J = 1.2$), 7.16 (1H, d, $J = 7.5$), 7.85 (1H, s, CH), 10.36 (1H, s, NH). Anal. Calcd for $C_{23}H_{22}ClN_2O_2$ (MW 393.89): C, 70.13; H, 5.63; N, 7.11. Found: C, 69.97; H, 5.49; N, 6.99.

18. Yield 38%, mp 138–140 °C. I.R.: 3250, 1701, 1614, 1235, 1132. ^{1}H NMR: 2.27 (3H, s, CH_3), 3.42 (3H, s, OCH_3), 3.59 (3H, s, OCH_3), 3.61 (3H, s, OCH_3), 3.65 (6H, s, 2 × OCH_3), 5.49 (2H, s, CH_2), 6.39 (1H, s, ox), 6.48 (2H, s, ph), 6.63 (1H, s, ox/ind), 6.80 (2H, s, ox/ind), 7.57 (1H, s, ind), 7.68 (1H, s, CH), 10.44 (1H, s, NH). Anal. Calcd for $C_{30}H_{29}ClN_2O_6$ (MW 549.02): C, 65.63; H, 5.32; N, 5.10. Found: C, 65.58; H, 5.30; N, 5.07.

19. Yield 70%, mp 190–193 dec °C. I.R.: 3298, 1675, 1598, 1240, 1122. ^{1}H NMR: 2.28 (3H, s, CH_3), 3.62 (6H, s, 2 × OCH_3), 3.67 (6H, s, 2 × OCH_3), 5.49 (2H, s, CH_2), 6.36 (1H, s, ox), 6.50 (2H, s, ph), 6.64(3H, m, ox + ind), 7.55 (1H, s, ind), 7.62 (1H, s, CH), 8.84 (1H, s, OH), 10.28 (1H, s, NH). Anal. Calcd for $C_{29}H_{27}ClN_2O_6$ (MW 534.99): C, 65.11; H, 5.09; N, 5.24. Found: C, 65.08; H, 6.20; N, 5.32.

20. Yield 32%, mp 152–154 °C. I.R.: 3582, 1698, 1609, 1092, 712. ^{1}H NMR: 2.12 (3H, s, CH_3), 2.26 (3H, s, CH_3), 3.17 (3H, s, OCH_3), 3.58 (3H, s, OCH_3), 3.61 (3H, s, OCH_3), 3.63 (6H, s, 2 × OCH_3), 5.48 (2H, s, CH_2), 6.36 (1H, s, ox), 6.47 (2H, s, ph), 6.66 (1H, s, ox/ind), 6.68 (1H, s, ox/ind), 7.56 (1H, s, ind), 7.60 (1H, s, CH), 10.36 (1H, s, NH). Anal. Calcd for $C_{31}H_{31}ClN_2O_6$ (MW 563.04): C, 66.13; H, 5.55; N, 4.98. Found: C, 66.10; H, 5.46; N, 5.20.

21. Yield 88%, mp 200 dec °C. I.R.: 3240, 1706, 1603, 1240, 1132. ^{1}H NMR: 2.25 (3H, s, CH_3), 3.61 (3H, s, OCH_3), 3.63 (3H, s, OCH_3), 3.66 (6H, s, 2 × OCH_3), 5.51 (2H, s, CH_2), 6.48 (2H, s, ph), 6.60 (1H, s, ind), 6.74 (1H, d, ox-4, $J = 2.0$), 6.90 (1H, d, ox-7, $J = 8.4$), 7.24 (1H, dd, ox-6, $J = 8.4$, $J = 2.0$), 7.60 (1H, s, ind), 7.77 (1H, s, CH), 10.76 (1H, s, NH). Anal. Calcd for $C_{29}H_{26}Cl_2N_2O_5$ (MW 553.44): C, 62.94; H, 4.74; N, 5.06. Found: C, 62.87; H, 4.69; N, 5.21.

22. Yield 40%, mp 250–255 °C. I.R.: 3211, 1696, 1660, 1045, 902. ^{1}H NMR: 2.23 (3H, s, CH_3), 3.60 (3H, s, OCH_3), 3.67 (6H, s, 2 × OCH_3), 3.77 (3H, s, OCH_3), 5.45 (2H, s, CH_2), 6.57 (2H, s, ph), 6.83 (1H, dd, ox-5/7, $J = 7.9$, $J = 0.9$), 6.91 (1H, s, ind), 7.03 (1H, dd, ox-5/7, $J = 7.9$, $J = 0.9$), 7.21 (1H, *t*, ox-6, $J = 7.9$), 7.47 (1H, s, ind), 8.43 (1H, s, CH), 10.79 (1H, s, NH). Anal. Calcd for $C_{29}H_{26}Cl_2N_2O_5$ (MW 553.44): C, 62.94; H, 4.74; N, 5.06. Found: C, 62.82; H, 4.78; N, 5.23.

23. Yield 61%, mp 190–192 dec °C. I.R.: 3170, 1675, 1603, 912, 733. ^{1}H NMR: 5.03 (2H, s, CH_2), 6.85 (2H, d, ph, $J = 4.0$), 6.97 (1H, d, $J = 7.8$), 7.25 (9H, m), 7.48 (1H, d, $J = 7.8$), 7.81 (1H, s, CH), 12.54 (1H, broad, NH). Anal. Calcd for $C_{24}H_{17}ClN_2O$ (MW 384.86): C, 74.90; H, 4.45; N, 7.28. Found: C, 74.49; H, 4.23; N, 6.99.

25. Yield 20%, mp 145–150 °C. I.R.: 3135, 1716, 1541, 1224, 753. ^{1}H NMR: 3.82 (3H, s, OCH_3), 6.90 (3H, m, ox), 7.23 (4H, m, ind), 7.62 (5H, m, ph), 7.97 (1H, s, CH), 10.72 (1H, s, NH). Anal. Calcd for $C_{24}H_{17}ClN_2O_2$ (MW 400.86): C, 71.91; H, 4.27; N, 6.99. Found: C, 72.03; H, 4.35; N, 7.05.

26. Yield 20%, mp 233–237 °C. I.R.: 3150, 1685, 1537, 1091, 697. ^{1}H NMR: 3.52 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 6.61 (1H, s, ox), 6.78 (2H, s, ox), 7.23 (4H, m, ind), 7.60 (5H, m, ph), 7.89 (1H, s, CH), 10.39 (1H, s, NH). Anal. Calcd for $C_{25}H_{20}N_2O_3$ (MW 396.44): C, 75.74; H, 5.08; N, 7.07. Found: C, 75.68; H, 6.97; N, 7.18.

27. Yield 20%, mp 145–150 °C. I.R.: 1706, 1619, 1235, 1091, 753. ^{1}H NMR: 3.27 (3H, s, CH_3), 3.82 (3H, s, OCH_3), 6.96 (1H, d, ox-4, $J = 2.2$), 7.09 (1H, d, ox-7, $J = 8.4$), 7.22 (4H, m, ind), 7.33 (1H, dd, ox-6, $J = 8.4$, $J = 2.2$), 7.63 (5H, m, ph), 8.05 (1H, s, CH). Anal. Calcd for $C_{25}H_{19}ClN_2O_2$ (MW 414.89): C, 72.37; H, 4.62; N, 6.75. Found: C, 72.56; H, 4.68; N, 6.58.

28. Yield 20%, mp 164–167 °C. I.R.: 3181, 1690, 1255, 1091, 773. ^{1}H NMR: 3.35 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 6.51 (1H, d, ox, $J = 8.8$), 6.85 (1H, d, ox, $J = 8.8$), 7.13 (3H, m, ind), 7.38 (1H, m, ind), 7.58 (5H, m, ph), 8.23 (1H, s, CH), 10.42 (1H, s, NH). Anal. Calcd for $C_{26}H_{22}N_2O_4$ (MW 426.47): C, 73.23; H, 5.20; N, 6.57. Found: C, 73.12; H, 5.11; N, 6.49.

*5.1.3. General procedure for the synthesis of compounds **3**, **6**, **7**, **9**, **12**, **13** and **17***

The appropriate oxindole **1** (5 mmol) was dissolved in acetic acid (25 mL) and treated with an equivalent of appropriate indole-3-carbaldehyde **2** and 37% hydrochloric acid (1 mL). The reaction mixture was refluxed for 20 h, the solid separated on cooling was collected by filtration. The crude products were purified by crystallization with ethanol to give the desired products, with the exception of compound **6** purified by column chromatography, with petroleum ether/acetone as the eluent.

3. Yield 70%, mp 200 dec °C. IR: 1596, 1572, 1299, 1260, 722. ^{1}H NMR: 3.79 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 6.59 (1H, d, ox, $J = 9.0$), 6.87 (1H, d, ox, $J = 9.0$), 7.13 (2H, m, ind), 7.35 (2H, m, ind), 8.00 (1H, s, CH), 10.45 (1H, s, NH), 12.47 (1H, broad, NH). Anal. Calcd for $C_{19}H_{15}ClN_2O_3$ (MW 354.79): C, 64.32; H, 4.26; N, 7.90. Found: C, 64.86; H, 4.11; N, 7.01.

6. Yield 25%, mp > 310 °C. I.R.: 3416, 1588, 1106, 722. ^{1}H NMR: 3.90 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 6.51 (1H, d, ox, $J = 8.8$), 6.65 (1H, d, ox, $J = 8.8$), 7.32 (1H, *t*, bzind, $J = 7.8$), 7.44 (1H, *t*, bzind, $J = 7.8$), 7.46 (1H, d, bzind, $J = 8.0$), 7.86 (1H, d, bzind, $J = 7.8$), 8.03 (1H, d, bzind, $J = 8.0$), 8.48 (1H, d, bzind, $J = 7.8$), 8.83 (1H, s, CH), 12.60 (2H, s, NH). Anal. Calcd for $C_{23}H_{17}ClN_2O_3$ (MW 404.85): C, 68.24; H, 4.23; N, 6.92. Found: C, 67.98; H, 4.01; N, 6.23.

7. Yield 25%, mp 272–280 dec °C. I.R.: 1603, 1567, 1286, 1061, 764. ^{1}H NMR: 3.44 (2H, broad, NH), 3.83 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 4.18 (3H, s, OCH_3), 7.06 (1H, s, ox), 7.46 (1H, *t*, ind, $J = 7.5$), 7.53 (1H, *t*, ind, $J = 7.5$), 7.69 (1H, d, ind, $J = 7.5$), 8.38 (1H, d, ind, $J = 7.5$), 9.52 (1H, s, CH). Anal. Calcd for $C_{20}H_{17}ClN_2O_4$ (MW 384.81): C, 62.42; H, 4.45; N, 7.28. Found: C, 62.03; H, 4.22; N, 6.99.

9. Yield 72%, mp 190–194 °C. I.R.: 3237, 1682, 1614, 1189, 733. ^{1}H NMR: 3.08 (2H, *t*, CH_2, $J = 7.2$), 4.57 (2H, *t*, CH_2, $J = 7.2$), 6.26 (1H, d, ox-4, $J = 2.2$), 6.60 (1H, dd, ox-6, $J = 8.3$, $J = 2.2$), 6.68 (1H, d, ox-7, $J = 8.3$), 7.20 (8H, m, ph + ind), 7.51 (1H, s, CH), 7.67 (1H, d, ind, $J = 8$), 8.81 (1H, s, OH), 10.29 (1H, s, NH). Anal. Calcd for $C_{25}H_{19}ClN_2O_2$ (MW 414.89): C, 72.37; H, 4.62; N, 6.75. Found: C, 72.78; H, 4.56; N, 6.43.

12. Yield 68%, mp 90–94 °C. I.R.: 3175, 1697, 1608, 1214, 745. ^{1}H NMR: 2.08 (2H, qui, CH_2, $J = 7.4$), 2.69 (2H, *t*, CH_2, $J = 7.4$), 4.39 (2H, *t*, CH_2, $J = 7.4$), 6.76 (2H, m, ox), 6.88 (1H, d, ox, $J = 7.5$), 7.25 (9H, m, ph + ind + ox), 7.63 (1H, s, CH), 7.65 (1H, d, ind, $J = 8.4$), 10.60 (1H, s, NH). Anal. Calcd for $C_{26}H_{21}ClN_2O$ (MW 412.91): C, 75.63; H, 5.13; N, 6.78. Found: C, 75.34; H, 4.97; N, 6.44.

13. Yield 73%, mp 203–205 °C. I.R.: 3391, 1711, 1604, 1158, 743. ^{1}H NMR: 1.98 (2H, qui, CH_2, $J = 7.4$), 2.67 (2H, *t*, CH_2, $J = 7.4$), 4.00 (2H, *t*, CH_2, $J = 7.4$), 7.20 (10H, m), 8.07 (3H, m) 11.98 (1H, s, NH). Anal. Calcd for $C_{26}H_{20}Cl_2N_2O$ (MW 447.36): C, 69.81; H, 4.51; N, 6.26. Found: C, 69.47; H, 4.29; N, 6.01.

17. Yield 25%, mp 170–175 °C. I.R.: 1696, 1598, 1296, 1209, 733. ^{1}H NMR: 3.35 (3H, s, OCH_3), 5.18 (2H, s, CH_2), 6.39 (1H, s, ox-4), 6.47 (2H, s, ox-6,7), 6.77 (2H, s, ind), 7.33 (8H, m, ph + ind + CH), 7.66 (1H, s, CH), 7.75 (1H, d, CH, $J = 8.2$), 10.45 (1H, s, NH). Anal. Calcd for $C_{27}H_{21}ClN_2O_2$ (MW 440.92): C, 73.55; H, 4.80; N, 6.35. Found: C, 73.38; H, 4.34; N, 6.70.

*5.1.4. Synthesis of compound **24***

The oxindole **1c** (7 mmol) was dissolved in toluene (30 mL) and treated with an equivalent of indole-3-carbaldehyde **2m** and *p*-toluenesulfonic acid (1 mmol). The reaction mixture was refluxed for 5 h, the solid separated on cooling was collected by filtration with a yield of 40%.

Mp 242–248 °C (ethanol). I.R.: 1598, 1562, 1148, 1086, 1004. ^{1}H NMR: 2.28 (3H, s, OCH_3), 7.10 (2H, d, $J = 8.0$), 7.47 (2H, d, $J = 8.0$), 7.67 (8H, m), 8.27 (1H, d, $J = 7.8$), 8.37 (1H, m), 9.97 (1H, s, NH). Anal. Calcd for $C_{24}H_{18}N_2O_2$ (MW 366.41): C, 78.67; H, 4.95; N, 7.65. Found: C, 78.56; H, 4.76; N, 7.32.

5.2. Biology

5.2.1. Cell-based screening assay

The NCI screening process occurs in two stages [27], beginning with the evaluation of all compounds against the 60 cell lines at 10^{-5} M. Compounds exhibiting significant growth inhibition are subsequently evaluated against the 60 cell lines at five concentration levels according to standard procedures (http://dtp.nci.nih.gov/branches/btb/ivclsp.html). In both cases, the exposure time is 48 h.

5.2.2. Cell culture, treatment, and biochemical determinations

The human leukemia cell line HL-60 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in air/5% CO_2. Solution of compound under test was prepared in DMSO at 10 mmol/L and diluted immediately before use in order to obtain the indicated concentration. In control cells, the corresponding amount of DMSO was added to the culture medium. At the end of the treatment, cells were collected and counted. Cell viability and cell survival were determined as described [28].

To determine cell cycle distribution [29], at the end of incubation, the cells were washed with phosphate buffered saline (PBS) and collected. The pellet was resuspended in 0.01% Nonidet P-40, 10 μg/mL RNase, 0.1% sodium citrate, and 50 μg/mL propidium iodide for 30 min at room temperature in the dark. Propidium iodide fluorescence was analyzed by using a Bryte flow cytometer (Biorad) and cell cycle analysis was performed using the Multicycle Cycle Phoenix Flow system, and Modfit 5.0 software.

Surface exposure of phosphatidylserine was measured with an Annexin V-FLUOS staining kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), as previously described [29].

In order to detect intracellular peroxides, the cells were incubated with 5 μM DCF-DA (Molecular Probes, Leiden, The Netherlands) for 30 min at 37 °C. Cells were analyzed acquiring the red fluorescence with a logarithmic amplification [30].

The activity of caspase enzymes hydrolyzing the peptide sequence DEVD, indicated as DEVDase activity, was measured in cell extracts by a fluorometric assay [31]. The cellular markers of autophagy were analyzed as described [32].

5.2.3. Intracellular content of free divalent cations

Intracellular content of free Mg^{2+} and Ca^{2+} were assessed according to Delva et al. [33], with minor modification. Briefly HL-60 cells treated and control were washed and resuspended at $2{,}5 \times 10^6$ cell/mL in RPMI1460 w/o FBS. To measure free Mg^{2+}, after incubation with the Mag-Fura2 fluorescent probe (5 μM) at 37 °C and in the dark for 60 min, the cells were washed twice and kept at room temperature for 45 min. The samples were analyzed by a fluorometric assay. Firstly the fluorescence emission at 510 nm was measured with alternate excitation at 335 nm (Mg bound dye) and 370 nm (free dye). Afterwards, 5 mmol/L EDTA and 5 mmol/L EGTA were added to the cuvette. The immediate (<10 s) change in fluorescence intensities at both wavelengths after the addition of EDTA and EGTA was considered for calculation of free resting Mg^{2+}. Triton X-100 was then added at a final concentration of 0.1% (v/v) to lyse the cells and determine the minimum fluorescence ratio, R_{min}. Subsequently, $MgSO_4$ (100 mM) was added to obtain the maximum fluorescence ratio, R_{max}. Intracellular-free Mg was measured in triplicate and calculated by using a $K_d = 2.1$ mM as follows:

$$[Mg^{2+}] = K_d\,(R - R_{min})\,Sf/(R_{max} - R)\,Sb$$

where R_{min} is the fluorescence ratio at 335/370 nm for uncomplexed Mag Fura2 (zero magnesium). R_{max} is the ratio of fluorescence at 335/370 nm for dye saturated with Mg^{2+}. Sf and Sb are the fluorescence intensities at 370 nm for Mag-Fura2 with zero Mg^{2+} and excess Mg^{2+}, respectively. R is the ratio of fluorescence at 335/370 nm of the sample to be measured.

Measurement of intracellular free Ca^{2+} was performed with the same protocol, but the cells were stained with INDO-1/AM probe at 5 μM concentration. The stained cells were analyzed exciting at 346 nm, and reading the emission at 485 nm and at 405 nm for the unbound and the bound dye respectively. Also in this analysis, the fluorescences were collected after adding to the samples EDTA, EGTA and digitonin (F_{min}) and $CaCl_2$ (F_{max}). The intracellular free Ca^{2+} concentration was evaluated by using a $K_d = 0.23$ mM and the following equation:

$$[Ca^{2+}] = K_d\,Q\,(R - R_{min})/(R_{max} - R_{min})$$

where Q is F_{min}/F_{max} at 485 nm, R is the fluorescence ratio at 405/485 of the samples, R_{min} is the fluorescence intensity ratio 405/485 after adding EDTA and EGTA (5 mM) and Digitonin (5 μM) and R_{max} is the fluorescence intensity ratio 405/485 after adding $CaCl_2$ (10 mM).

Author contributions

The authors contributed equally to this work.

Acknowledgements

This work has been supported by a grant from the University of Bologna, Italy (RFO 2011), and Tavola Valdese Ufficio 8PM. We are grateful to the National Cancer Institute (Bethesda, MD) for the anticancer tests and to the Centro Interdipartimentale Ricerche Biotecnologiche (CIRB) of the University of Bologna for the flow cytometric facility.

References

[1] A. Andreani, A. Locatelli, M. Rambaldi, A. Leoni, R. Bossa, A. Fraccari, I. Galatulas, Potential antitumor agents. 25. Synthesis and cytotoxic activity of 3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones, Anticancer Research 16 (1996) 3585–3588.

[2] A. Andreani, A. Locatelli, A. Leoni, R. Morigi, M. Chiericozzi, A. Fraccari, I. Galatulas, G. Salvatore, Synthesis and potential coanthracyclinic activity of pyridylmethylene and indolylmethylene lactams, European Journal of Medicinal Chemistry 33 (1998) 905–909.

[3] A. Andreani, M. Granaiola, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, V. Garaliene, Synthesis and antitumor activity of 1,5,6-substituted 3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones, Journal of Medicinal Chemistry 45 (2002) 2666–2669.

[4] A. Andreani, M. Granaiola, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, V. Garaliene, G. Farruggia, L. Masotti, Substituted *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones with antitumor activity, Bioorganic & Medicinal Chemistry 12 (2004) 1121–1128.

[5] A. Andreani, S. Burnelli, M. Granaiola, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, L. Varoli, N. Calonghi, C. Cappadone, G. Farruggia, M. Zini, C. Stefanelli, L. Masotti, Substituted *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones with antitumor activity. Effect on the cell cycle and apoptosis, Journal of Medicinal Chemistry 50 (2007) 3167–3172.

[6] A. Andreani, S. Bellini, S. Burnelli, M. Granaiola, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, L. Varoli, N. Calonghi, C. Cappadone, M. Zini, C. Stefanelli, L. Masotti, R.H. Shoemaker, Substituted *E*-3-(3-indolylmethylene)1,3-dihydroindol-2-ones with antitumor activity. In-depth study of the effect on growth of breast cancer cells, Journal of Medicinal Chemistry 53 (2010) 5567–5575.

[7] A. Andreani, A. Locatelli, A. Leoni, M. Rambaldi, R. Morigi, R. Bossa, M. Chiericozzi, A. Fraccari, I. Galatulas, Synthesis and potential coanthracyclinic activity of substituted 3-(5-imidazo[2,1-b]thiazolylmethylene)-2-indolinones, European Journal of Medicinal Chemistry 32 (1997) 919–924.
[8] A. Andreani, M. Granaiola, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, G. Giorgi, L. Salvini, V. Garaliene, Synthesis and antitumor activity of substituted 3-(5-imidazo[2,1-b]thiazolylmethylene)-2-indolinones, Anticancer Drug Design 16 (2001) 167–174.
[9] L.K. Mehta, J. Parrick, F. Payne, Preparation of 3-ethyloxindole-4,7-quinone, Journal of Chemical Research, Synopses (1998) 190–191.
[10] S.-I. Sakai, N. Aimi, A. Kubo, M. Kitagawa, M. Hanasawa, K. Katano, K. Yamaguchi, J. Haginawa, Structure of Gardneramine and 18-demethylgardneramine, Chemical & Pharmaceutical Bulletin 23 (1975) 2805–2817.
[11] R.J.S. Beer, H.F. Davenport, A. Robertson, Extensions of the synthesis of hydroxyindoles from p-benzoquinones, Journal of the Chemical Society (1953) 1262–1264.
[12] C.F. Koelsch, A synthesis of ethyl quininate from m-cresol, Journal of the American Chemical Society 66 (1944) 2019–2020.
[13] A. Andreani, M. Rambaldi, D. Bonazzi, L. Greci, F. Andreani, Potential antitumor agents. III. Hydrazone derivatives of 5-substituted 2-chloro-3-formyl-6-methylindole, Farmaco 34 (1979) 132–138.
[14] A. Romeo, H. Corrodi, E. Hardegger, Umsetzungen des o-nitrophenylessigesters und des 2-chlor-6-nitro-phenyl-brenztraubensäureesters, Helvetica Chimica Acta 38 (1955) 463–467.
[15] C. Crestini, R. Saladino, New efficient and mild synthesis of 2-oxindoles by one-pot Wolff-Kishner like reduction of isatin derivatives, Synthetic Communications 24 (1994) 2835–2842.
[16] R. Huisgen, H. Konig, A.R. Lepley, Nucleophilic aromatic substitutions. XVIII. New ring closures through arynes, Chemische Berichte 93 (1960) 1496–1506.
[17] K.E. Schulte, J. Reisch, U. Stoess, Chloroformylation of α-pyrrolones, Angewandte Chemie International Edition 4 (1965) 1081–1082.
[18] R. Gatti, V. Cavrini, P. Roveri, F. Bianucci, P. Legnani, Sintesi ed attività antibatterica in vitro di N-metilnitroni e nitrovinili derivati da alcune 2-cloroindol-3-carbossialdeidi N-sostituite, Farmaco 36 (1981) 102–108.
[19] A. Andreani, M. Rambaldi, A. Locatelli, M. Conti, S. Malandrino, Synthesis and antiinflammatory activity of indole carboxylic acids and esters, Acta Pharmaceutica Nordica 3 (1991) 5–8.
[20] Hoechst AG Patent: DE2707268, Chemical Abstract. vol. 89, 1978, 179858.
[21] O. Kepp, L. Galluzzi, M. Lipinski, J. Yuan, G. Kroemer, Cell death assays for drug discovery, Nature Reviews Drug Discovery 10 (2011) 221–237.
[22] A. Notte, L. Leclere, C. Michiels, Autophagy as a mediator of chemotherapy induced cell death in cancer, Biochemical Pharmacology 82 (2011) 427–434.
[23] S. Barth, D. Glick, K.F. Macleod, Autophagy: assays and artifacts, Journal of Pathology 221 (2010) 117–124.
[24] S. Orrenius, P. Nicotera, B. Zhivotovsky, Cell death mechanisms and their implications in toxicology, Toxicological Sciences 119 (2011) 3–19.
[25] F.I. Wolf, V. Trapani, Magnesium and its transporters in cancer: a novel paradigm in tumour development, Clinical Science (London) 123 (2012) 417–427.
[26] A.M. Woodward, M. Senchyna, P. Argüeso, Differential contribution of hypertonic electrolytes to corneal epithelial dysfunction, Experimental Eye Research 100 (2012) 98–100.
[27] A. Monks, D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A. Vaigro-Wolff, M. Gray-Goodrich, H. Campbell, J. Mayo, M. Boyd, Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines, Journal of the National Cancer Institute 83 (1991) 757–766.
[28] M. Zini, C.L. Passariello, D. Gottardi, S. Cetrullo, F. Flamigni, C. Pignatti, A. Minarini, V. Tumiatti, A. Milelli, C. Melchiorre, C. Stefanelli, Cytotoxicity of methoctramine and methoctramine-related polyamines, Chemico-Biological Interactions 181 (2009) 409–416.
[29] A. Andreani, M. Granaiola, A. Locatelli, R. Morigi, M. Rambaldi, L. Varoli, N. Calonghi, C. Cappadone, G. Farruggia, C. Stefanelli, L. Masotti, T.L. Nguyen, E. Hamel, R.H. Shoemaker, Substituted 3-(5-imidazo[2,1-b]thiazolylmethylene)-2-indolinones and analogues: synthesis, cytotoxic activity, and study of the mechanism of action, Journal of Medicinal Chemistry 55 (2012) 2078–2088.
[30] A. Andreani, S. Burnelli, M. Granaiola, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, L. Varoli, N. Calonghi, C. Cappadone, G. Farruggia, M. Zini, C. Stefanelli, L. Masotti, N.S. Radin, R.H. Shoemaker, New antitumor imidazo[2,1-b]thiazole guanylhydrazones and analogues, Journal of Medicinal Chemistry 51 (2008) 809–816.
[31] C. Muscari, F. Bonafé, I. Stanic, F. Flamigni, C. Stefanelli, G. Farruggia, C. Guarnieri, C.M. Caldarera, Polyamine depletion reduces TNFα/MG132-induced apoptosis in bone marrow stromal cells, Stem Cells 23 (2005) 983–991.
[32] A. Minarini, M. Zini, A. Milelli, V. Tumiatti, C. Marchetti, B. Nicolini, M. Falconi, G. Farruggia, C. Cappadone, C. Stefanelli, Synthetic polyamines activating autophagy: effects on cancer cell death, European Journal of Medicinal Chemistry 67 (2013) 359–366.
[33] P. Delva, M. Degan, M. Trettene, A. Lechi, Insulin and glucose mediate opposite intracellular ionized magnesium variations in human lymphocytes, Journal of Endocrinology 190 (2006) 711–718.