Articles

Substituted *E*-3-(2-Chloro-3-indolylmethylene)1,3-dihydroindol-2-ones with Antitumor Activity. Effect on the Cell Cycle and Apoptosis[1]

Aldo Andreani,*,† Silvia Burnelli,† Massimiliano Granaiola,† Alberto Leoni,† Alessandra Locatelli,† Rita Morigi,† Mirella Rambaldi,† Lucilla Varoli,† Natalia Calonghi,‡ Concettina Cappadone,‡ Giovanna Farruggia,‡ Maddalena Zini,‡ Claudio Stefanelli,‡ and Lanfranco Masotti‡

Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy, Dipartimento di Biochimica "G. Moruzzi", Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

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The synthesis and antitumor activity of new *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones is described. They were studied at the National Cancer Institute, taking into consideration the 50% growth inhibitory power (pGI_{50}), the cytostatic effect (pTGI = total growth inhibition), and the cytotoxic effect (pLC_{50}). All the compounds were potent growth inhibitors, with mean pGI_{50} ranging from 5.26 to 7.72. They were also analyzed with NCI COMPARE algorithm. Further studies were dedicated to the effects on the cell cycle and apoptosis.

Introduction

We describe synthesis and antitumor activity of a new series of *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones (**3a**−**p**, see Scheme 1 and Table 1). In our previous work, we have shown that in the most active compounds the chloroindole portion should bear a methoxy group at the 5 position and a methyl group at the 6 position; moreover, at least one of the two NH groups should be unsubstituted.[2−5]

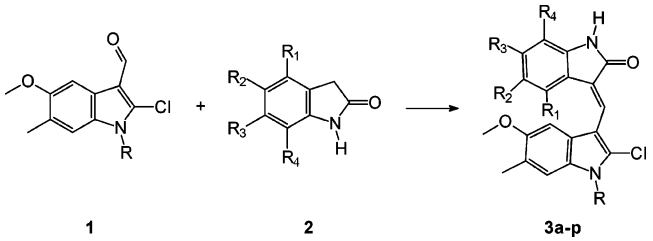
In the last paper of this series,[5] we noticed that a further increment of activity could be obtained with the introduction, in the indolinone portion, of a substituent at the 4/5 position or with a disubstitution at the 5 and 6 positions. In such a case, the activity was maintained even when the indolinone NH is replaced by a *N*-methyl group. Compounds endowed with significant antitumor activity were also obtained by introducing a benzyl or 4-chlorobenzyl group at the nitrogen of 2-chloro-5-methoxy-6-methyl-indole.

In this paper, we describe the synthesis of new analogs (**3i**−**l**) bearing the aforementioned 4-chlorobenzyl derivative as the chloroindole portion and, in the indolinone portion, the same substituents tested in the previous paper.[5] Moreover, the effect of the following features was also studied: (1) introduction of fluorine at the 5 position of the indolinone (**3a**,**e**,**h**); (2) introduction of a methyl group on the chloroindole nitrogen (**3b**−**g**,**n**); and (3) introduction of a condensed benzene ring in the 6,7 position of the indolinone portion (**3m**−**p**).

Chemistry

The reaction between a 2-chloroindolaldehyde **1** and the equivalent of an oxindole **2** was performed in methanol in the presence of piperidine (see Scheme 1 and Table 1). Only compound **3b** was prepared in water in the presence of sodium carbonate and glycine. All compounds **3** were obtained as pure geometrical isomers. The NMR spectra (see Supporting Information, Table S1) are in agreement with those previously published for analogous *E*-derivatives. Nevertheless, some NOE experiments were performed on compound **3d**. Irradiation of the methyl group at the 6 position of chloroindole (2.32 ppm) gave NOE at 7.51 ppm (ind-7), which allowed the attribution of the singlet at 6.49 ppm to ind-4. When ind-4 was irradiated, NOE was observed at 3.59 (OCH_3), 7.74 (CH), and 6.74 ppm (ox-4): this last NOE effect is compatible only with the *E*-configuration. A feature common to all compounds **3** is the hydrogen at position 4 in the 2-chloroaldehydes (7.4−7.6 ppm in compounds **1**), which is shielded in the *E*-isomers of Knoevenagel adducts (6.4−6.9 ppm for the same hydrogen in compounds **3**).

Scheme 1[a]



[a] For R−R_4, see Table 1.

Biology

(a) Antitumor Activity.[6] As a primary screening, compounds **3a**−**p** were submitted to the Developmental Therapeutics Program (DTP[a]) at the National Cancer Institute (NCI) for evaluation of antitumor activity in the human cell line screen. In a preliminary screen at a single concentration (100 μM)

* To whom correspondence should be addressed. Tel.: +39-051-2099714. Fax: +39-051-2099734. E-mail: aldo.andreani@unibo.it.
† Dipartimento di Scienze Farmaceutiche, Università di Bologna.
‡ Dipartimento di Biochimica "G. Moruzzi", Università di Bologna.

[a] Abbreviations: DTP, Developmental Therapeutics Program; NCI, National Cancer Institute; GI, growth inhibition; TGI, total growth inhibition; LC, lethal concentration; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DABCO, 1,4-diazabicyclo-[2,2,2]octane.

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Table 1. Compounds **3a**−**p**[a]

cmpd	R	R_1	R_2	R_3	R_4	formula	MW	mp, °C
3a	H	H	F	H	H	$C_{19}H_{14}ClFN_2O_2$	356.78	236−240 dec
3b	CH_3	H	H	H	H	$C_{20}H_{17}ClN_2O_2$	352.82	215−217 dec
3c	CH_3	Cl	H	H	H	$C_{20}H_{16}Cl_2N_2O_2$	387.26	205−207
3d	CH_3	H	Cl	H	H	$C_{20}H_{16}Cl_2N_2O_2$	387.26	240−242 dec
3e	CH_3	H	F	H	H	$C_{20}H_{16}ClFN_2O_2$	370.81	277−280 dec
3f	CH_3	H	OH	H	H	$C_{20}H_{17}ClN_2O_3$	368.82	292−295 dec
3g	CH_3	H	OH	CH_3	H	$C_{21}H_{19}ClN_2O_3$	382.84	296−300 dec
3h	Bn	H	F	H	H	$C_{26}H_{20}ClFN_2O_2$	446.90	250−252 dec
3i	4Cl-Bn	Cl	H	H	H	$C_{26}H_{19}Cl_3N_2O_2$	497.80	252−255 dec
3j	4Cl-Bn	H	Cl	H	H	$C_{26}H_{19}Cl_3N_2O_2$	497.80	236−240 dec
3k	4Cl-Bn	H	OH	H	H	$C_{26}H_{20}Cl_2N_2O_3$	479.36	180−183
3l	4Cl-Bn	H	OH	CH_3	H	$C_{27}H_{22}Cl_2N_2O_3$	493.38	287−290 dec
3m	H	H	H	CBR		$C_{23}H_{17}ClN_2O_2$	388.85	317−320 dec
3n	CH_3	H	H	CBR		$C_{24}H_{19}ClN_2O_2$	402.87	302−305 dec
3o	Bn	H	H	CBR		$C_{30}H_{23}ClN_2O_2$	478.97	308−310 dec
3p	4Cl-Bn	H	H	CBR		$C_{30}H_{22}Cl_2N_2O_2$	513.42	292−295 dec

[a] Bn = benzyl, CBR = condensed benzene ring.

Table 2. Sixty Cell Panel: Growth Inhibition, Cytostatic, and Cytotoxic Activity of the Selected Compounds

cmpd[a]	modes	leukemia	NSCLC	colon	CNS	melanoma	ovarian	renal	prostate	breast	MG-MID[b]
3a	pGI_{50}	6.92	6.55	6.34	6.62	6.46	6.37	6.66	6.04	6.43	6.52
	pTGI	5.48	5.29	5.33	5.40	5.16	5.38	5.17	5.50	5.58	5.34
	pLC_{50}	4.03	4.33	4.63	4.27	4.43	4.27	4.39	4.34	4.27	4.34
3b	pGI_{50}	7.52	7.45	7.04	7.03	6.87	7.34	6.98	6.09	6.63	7.09
	pTGI	5.62	5.47	5.97	4.88	5.21	5.39	5.36	5.23	5.92	5.48
	pLC_{50}		4.22	4.65	4.20	4.33	4.20	4.39	4.36	4.24	4.29
3c	pGI_{50}	5.43	5.36	5.51	5.15	5.20	5.00	5.22	5.50	5.17	5.27
	pTGI	4.87	4.58	4.96	4.75	4.72	4.50	4.73	4.96	4.64	4.72
	pLC_{50}	4.40	4.35	4.42	4.40	4.44	4.33	4.40	4.55	4.33	4.39
3d	pGI_{50}	6.17	6.27	6.15	6.36	6.39	6.06	6.90	6.05	6.43	6.35
	pTGI	5.42	5.57	5.44	5.60	5.40	5.44	5.84	5.49	5.72	5.56
	pLC_{50}	4.07	4.96	4.91	4.95	4.91	4.95	5.26	4.97	5.06	4.92
3e	pGI_{50}	6.77	6.77	7.03	6.97	6.93	6.65	6.66	7.39	7.22	6.90
	pTGI	6.44	6.03	6.44	6.26	5.93	5.90	5.93	6.54	6.52	6.18
	pLC_{50}	5.91	5.22	5.79	5.41	5.24	4.95	5.28	6.03	6.01	5.48
3f	pGI_{50}	8.00	7.59	7.88	8.00	7.49	7.54	8.00	7.69	7.44	7.72
	pTGI	5.89	5.49	6.08	5.52	5.70	4.98	4.72	4.75	5.82	5.49
	pLC_{50}		4.15	4.61		4.51		4.09		4.04	4.19
3g	pGI_{50}	7.36	6.53	6.62	6.68	6.42	6.76	6.34	6.07	6.69	6.63
	pTGI	5.98	4.96	5.33	4.76	4.83	4.65	4.58	5.20	5.18	5.02
	pLC_{50}	4.04	4.17	4.41		4.08					4.09
3h	pGI_{50}	5.35	5.71	5.72	5.65	5.88	5.69	5.85	5.73	5.02	5.71
	pTGI	4.17	5.27	5.09	5.26	5.34	5.34	5.44	5.40	5.35	5.18
	pLC_{50}		4.65	4.56	4.49	4.70	4.72	5.03	5.07	4.79	4.65
3i	pGI_{50}	5.39	5.21	5.29	5.20	5.39	5.27	5.17	5.40	5.32	5.28
	pTGI	4.61	4.63	4.63	4.68	4.78	4.41	4.70	4.82	4.68	4.66
	pLC_{50}	4.07	4.12	4.16	4.25	4.30	4.11	4.15	4.35	4.18	4.18
3j	pGI_{50}	5.04	5.25	5.20	5.56	5.35	5.36	5.05	5.63	5.21	5.26
	pTGI	4.65	4.64	4.47	5.09	4.78	4.69	4.65	5.22	4.59	4.71
	pLC_{50}	4.27	4.09	4.10	4.39	4.13	4.08	4.15	4.72	4.18	4.19
3k	pGI_{50}	5.45	6.08	6.79	6.90	6.20	6.38	6.46	5.44	6.40	6.33
	pTGI	5.00	4.91	4.95	5.15	4.69	4.94	4.82	4.67	5.01	4.91
	pLC_{50}	4.23	4.21	4.25	4.25	4.15	4.30	4.16		4.32	4.22
3l	pGI_{50}	5.45	5.46	5.83	5.67	5.68	5.45	5.57	5.04	5.74	5.59
	pTGI	4.33	4.31	4.47	4.30	4.54	4.22	4.24		4.43	4.35
	pLC_{50}		4.06	4.09		4.17					4.04
3m	pGI_{50}	7.99	6.99	7.77	7.87	8.00	7.92	7.05	7.77	7.40	7.56
	pTGI	6.27	5.37	5.43	6.10	5.45	6.15	4.51	4.30	5.69	5.51
	pLC_{50}	4.44	4.31	4.30	4.30	4.39	4.32	4.30	4.30	4.30	4.33
3n	pGI_{50}	7.76	7.51	7.11	6.94	7.27	7.45	7.14	6.25	7.33	7.26
	pTGI	6.30	5.32	6.89	5.79	5.18	6.25	5.58	5.61	5.52	5.79
	pLC_{50}	4.07	4.30	5.29	4.51	4.44	4.64	4.65	4.59	4.86	4.61
vincristine-sulfate	pGI_{50}	7.00	6.60	7.00	6.90	6.80	6.50	6.50	6.90	6.50	6.70
	pTGI	4.80	4.80	5.40	5.20	5.10	4.70	4.70	5.20	5.10	5.00
	pLC_{50}	3.20	3.60	4.10	3.70	3.60	3.50	3.60	3.50	3.50	3.60

[a] Highest concn = 10^{-4} M, except for vincristine (10^{-3} M); only modes showing a value >4.00 are reported. [b] Mean graph midpoint, that is, the calculated mean panel.

against three human cell lines (NCI-H460 lung cancer, MCF7 breast cancer, and SF-268 glioma) a compound is considered active when it reduces the growth of any of the cell lines to 32% or less. Fourteen of the compounds reported in Scheme 1 were active and passed on for evaluation in the full panel of 60 human tumor cell lines. This panel is organized into subpanels representing leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and central nervous system.

The test compounds were dissolved in DMSO and evaluated using five concentrations at 10-fold dilutions, the highest being 10^{-4} M. Table 2 reports the results obtained, expressed as −log of the molar concentration, taking into consideration the 50% growth inhibitory power (pGI_{50}), the cytostatic effect (pTGI = total growth inhibition) and the cytotoxic effect (pLC_{50}). Some of the compounds were reassayed based on the results of the first screen: compounds **3c**, **3h**, **3i**, and **3j** were assayed once, all other compounds were assayed twice. All the compounds were potent growth inhibitors with mean pGI_{50} ranging from 5.26 to 7.72. Nine of them (**3a**,**b**,**d**−**g**,**k**,**m**,**n**) were submitted to BEC (Biological Evaluation Committee of the NCI) for a possible future development. The average potency, in all 60 cell lines, of the compounds at the GI_{50} endpoint of the screen ranged across about 2.5 log units. The most potent was **3f** with an average pGI_{50} value of 7.72 across the 60 cell lines; the least potent was **3j** with an average pGI_{50} value of 5.26. At the TGI endpoint, the range of potencies was similar, about 1.8 log units: from an average pTGI for **3e** of 6.18 to an average pTGI value of 4.35 for **3l**.

The screening data for all 14 compounds (comprising 24 individual assays including the data from repeated screens) was analyzed using the DTP COMPARE algorithm (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html). This analysis, based on the patterns of relative potencies of the compounds among the cell lines in the screen, can select compounds previously submitted to the screening assay (approximately 80 000 DTP compounds are in the public domain), which might act by the same mechanisms as the indole derivatives described in this paper.[7]

For the compounds in this study the top 200 COMPARE matches for each individual compound were collated to look for commonalities. As a group, the compounds **3m**, **3a**, **3b**, **3d**, **3n**, and **3f** showed the greatest number of commonalities to a subset of public compounds at the GI_{50} endpoint. For the 18 public compounds, at least five of the six above-mentioned compounds had average correlations of 0.56 (see Supporting Information, Table S3). While this correlation value would be at the low end of significance for an individual compound,[8] these compounds are reasonable "hits" from the COMPARE analysis because of their appearance in multiple sets of results. The correlations were driven largely by the differential responses within the melanoma, ovarian, renal, and breast cancer panels. The other compounds described in this paper did not show commonalities in COMPARE analysis at the GI_{50} endpoint, either to the six compounds listed above or to any compounds with known mechanisms of action.

Of these 18 public compounds, several are known high molecular weight agents that have been evaluated in clinical trials. Halichondrin B,[9,10] homohalichondrin B aldehyde,[11,12] and dolastatin 10[13,14] are marine natural products, antimitotic agents that inhibit microtubule assembly and cause induction of apoptosis. COMPARE also identified compounds that are analogs of colchicine, another known mitotic inhibitor. A synthetic analog of halichondrin B entered phase 3 clinical trials in the fourth quarter of 2006, and a synthetic analog of dolastatin 10 is in phase 2 clinical trials. The remaining compounds are small molecules.

At the TGI endpoint, the group of compounds **3a**, **3b**, **3k**, and **3g** showed the most consistent commonalities to a number of public compounds, with an average COMPARE correlation of about 0.60 (see Supporting Information, Table S4). The results were qualitatively similar to those at the GI_{50} endpoint in that the known compounds identified by COMPARE were

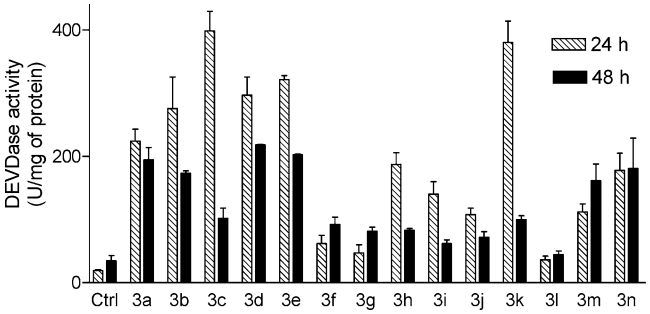

Figure 1. Activation of caspase protease. Caspase acting on the peptide sequence DEVD (DEVDase activity) was measured in IGROV-1 cells treated for 24 and 48 h with the compound under testing (5 μM). Data represent the means ± SEM of triplicate determinations.

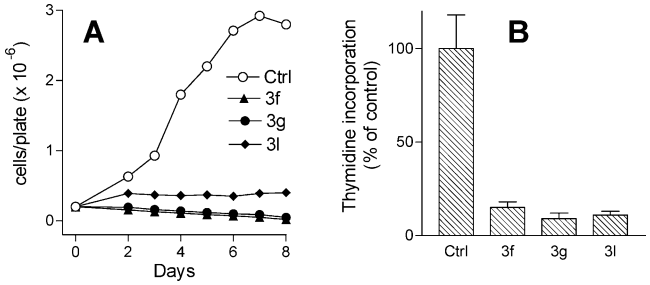

Figure 2. Effect of compounds **3f**, **3g**, and **3l** (5 μM) on IGROV-1 cell proliferation. (A) Rate of cell growth determined as total cell number. The panels depict the results obtained in one experiment repeated twice with comparable results. (B) Rate of DNA synthesis, measured by thymidine incorporation, in cells incubated for 24 h in the presence of the compound under testing. Data are means ± SEM of triplicate measurements.

tubulin-interacting agents: combretastatin A4 and vincristine. The other identified compounds were all small molecules. In contrast to the results at the GI_{50} endpoint, these correlations were driven by differential responses within all of the cell panels, and many of the individual correlations were above 0.6 and would stand on their own. The larger correlations of the cell line patterns at the TGI endpoint, compared to the GI_{50} endpoint, are a previously observed characteristic of tubulin-interacting agents.[8]

(b) Effects on Cell Death and Growth of Ovarian Carcinoma Cells. To give some insights into the biological effects of the novel compounds, we first determined whether they induced apoptosis. The ovarian carcinoma cell line IGROV-1 was used in these experiments. The cells were treated with a 5 μM concentration of the compounds, and after 24 and 48 h, we determined the activation of effector caspase proteases 3 and 7 acting on the substrate sequence Asp-Glu-Val-Asp (DEVD). Activation of these caspases represents a marker of apoptotic cell death.[15] Figure 1 shows that all the compounds triggered caspase activation, but the degree and timing of activation is different from one compound to another. However, a low degree of caspase activation was observed with **3f**, **3g**, and especially **3l**, accordingly, to their low cytotoxicity against ovarian tumors observed in the 60 cell panel. Similar results were obtained in experiments in which leukemia HL60 cells were used (data not shown). On the other hand, growth analysis of cells treated with **3f**, **3g**, and **3l** showed that these compounds produced strong cytostatic effect (Figure 2A). The mitotic index was also evaluated by measuring DNA synthesis by thymidine incorporation assay. Figure 2B shows that treatment with the derivatives **3f**, **3g**, and **3l** caused a reduction of thymidine incorporation by more than 85% with respect to control. It is worth noting that the cytostatic effect of these compounds is

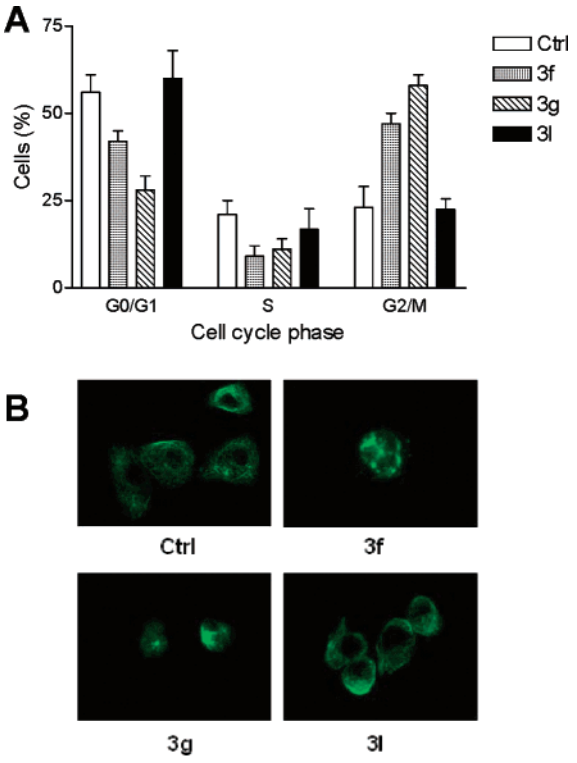

Figure 3. Effects of compounds **3f**, **3g**, and **3l** on cell cycle in IGROV-1 cells. (A) Cells were treated with 5 μM of each compound for 24 h, and afterward, cell cycle distribution was determined. Results are means ± SEM of three determinations. (B) Analysis of tubulin polymerization level by fluorescence microscopy.

irreversible because it persists after their withdrawal from the medium following a 24 h period of cell exposure (not shown).

To assess whether compounds **3f**, **3g**, and **3l** interfered with the cell cycle progression, the cells were incubated for 24 h in the presence of these compounds, and the analysis of DNA profiles was performed by flow cytometry. Figure 3A shows that **3f** and **3g** caused the accumulation of IGROV-1 cells in the G2/M phase, whereas the majority of control cells were in the G0/G1 phase of the cell cycle, and less than 25% were in the G2/M phase. On the other hand, in cells treated with **3l**, a similar block of cell cycle was not observed.

The images obtained by immunofluorescence microscopy analysis of tubulin polymerization (Figure 3B) confirmed that **3f** and **3g** arrested cells in mitosis, indicated by the condensation of tubulin, marker of cytoskeleton reorganization. No significant effect on tubulin polymerization was seen after **3l** treatment, indicating that this compound does not interfere with microtubule dynamics.

Discussion

The introduction of a substituent at the positions 4, 5, and 6 of the oxindole portion in the 4-chlorobenzyl derivatives (**3i**−**l**) gave mainly rise to a slight loss of activity in comparison to the unsubstituted derivatives:[5] nevertheless, the growth inhibition was still relevant with mean pGI_{50} values ranging from 5.28 to 6.33. Among those compounds, it is interesting to point out that the 5-hydroxy derivative **3k**, while was not significantly different in the mean pGI_{50} (6.33) with respect to the unsubstituted derivative (6.44),[5] showed a potent growth inhibition toward selected cell lines (pGI_{50} 6.79 colon and 6.90 CNS), and this was the reason why it was selected by BEC. Compound **3k** also rapidly activated apoptosis in ovarian carcinoma cells. Interestingly, the addition of a methyl group at the 6 position, leading to **3l**, significantly decreased the antiproliferative power and abolished the proapoptotic action and cytotoxicity.

The introduction of fluorine at the 5 position of the indolinone portion (**3a**) led to a slight loss of activity if compared to the unsubstituted analog[4] but with a clearer selectivity toward leukemic cells.

The introduction of a methyl group at the chloroindole nitrogen when the oxindole portion is unsubstituted (**3b**) led to an equipotent agent (mean pGI_{50} 7.09) with respect to the unsubstituted analog[4] (7.00), whereas a strong increase in growth inhibition was observed in comparison to the benzyl analog, which did not pass the three cell line test.[5]

Even the comparison between **3b** (mean pGI_{50} 7.09) and the 5-halogenated analogs (**3d** and **3e**) shows a decrease in activity. Compound **3e** was the most selective among the three compounds considered (pGI_{50} 7.03, colon; 7.39, prostate; and 7.22, breast) but showed even the highest toxicity among all the compounds described in this paper.

On the contrary, the introduction of fluorine in one of the benzyl derivatives (**3h**) led to a considerable increase in activity because the unsubstituted analog did not pass the three cell line test.[5]

Compounds **3a**, **3b**, and **3e** were submitted to BEC because they were selective (**3a**, **3e**) or endowed with low cytotoxicity (**3b**, mean pLC_{50} 4.29).

When chlorine is present at the 4 position of the indolinone portion (**3c**), the introduction of a methyl group in the chloroindole portion led to an almost equipotent agent compared to the unsubstituted analog and to the benzyl derivative,[5] but in all the other derivatives (**3d**−**g**) led to a strong increase in activity with respect to the unsubstituted as well as the benzyl analogs reported in this paper (see, for example, **3e** versus **3a** and **3h**) or in the previous one.[5] However, the introduction of a methyl group at the 6 position of compound **3f**, leading to **3g**, decreased the mean pGI_{50}.

All the above-mentioned derivatives (**3d**−**g**) were selected by BEC, and **3f** resulted in the most active compound of the whole series (mean pGI_{50} 7.72) with low cytotoxicity (mean LC_{50} 4.19). The antitumor action of **3f** in ovarian carcinoma cells was correlated to the ability to block cell cycle progression leading to accumulation in the G2/M phase. The presence of a polar hydroxy group at the 5 position could be responsible for the hydrogen bonding with its target.

The bulky benzoindole system resulted in a convenient pharmacophoric group in this class of compounds but apparently only within a given molecular dimension, because compounds bearing a benzyl ring at the chloroindole nitrogen (**3o**,**p**) did not show antitumor activity, whereas the unsubstituted analogs **3m**,**n** gave mean pGI_{50} values of 7.56 (mean pLC_{50} 4.33) and 7.26 (mean pLC_{50} 4.61), respectively, and were selected by BEC.

Conclusion

Biological data indicate that the growth-inhibiting activity of the compounds investigated was associated with both cytotoxic and cytostatic effects. Most of the compounds triggered apoptosis, as shown by caspase activation, at least in ovarian carcinoma and leukemia cells. Experiments with IGROV-1 cells showed that compounds with low cytotoxicity (**3f**, **3g**) can interfere with cell cycle progression. On the other hand, compound **3l** appeared to trigger a different, not yet characterized, biochemical pathway. These observations suggest that the described compounds can interfere with cancer cell proliferation by multiple mechanisms.[16−20]

Experimental Section

(a) Chemistry. The melting points are uncorrected. Analyses (C, H, N) were within ±0.4% of the theoretical values. Bakerflex plates (silica gel IB2-F) were used for TLC; the eluent was 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 Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz (see Supporting Information, Table S1).

The synthesis of a new aldehyde is reported below. All the other starting compounds are commercially available (2-indolinone and 5-chloro-2-indolinone), otherwise, they are previously described 2-indolinones (5-fluoro,[21] 4-chloro,[22] 5-hydroxy,[23] 5-hydroxy-6-methyl,[4] and 6,7-benzo, i.e., 1,3-dihydro-2*H*-benzo[*g*]indol-2-one)[24] or aldehydes (2-chloro-5-methoxy-6-methylindole-3-carbaldehyde,[25] 1-benzyl-2-chloro-5-methoxy-6-methylindole-3-carbaldehyde,[5] and 2-chloro-1-*p*-chlorobenzyl-5-methoxy-6-methylindole-3-carbaldehyde).[5]

2-Chloro-5-methoxy-1,6-dimethylindole-3-carbaldehyde. 2-Chloro-5-methoxy-6-methylindole-3-carbaldehyde (2 mmol) was dissolved in acetone (20 mL). Anhydrous potassium carbonate (3 mmol) was added and the mixture treated portionwise, under stirring, with dimethyl sulfate (5 mmol). Stirring was maintained for 10 min at room temperature and under reflux for 1 h. Potassium carbonate was removed by filtration and the solvent was removed by low-pressure evaporation. The residue was crystallized from petroleum ether with a yield of 75%: $C_{12}H_{12}ClNO_2$, MW 237.68; mp 180−183 °C; IR ν_{max} (cm^{-1}): 1650, 1044, 964, 856, 683; ^1H NMR δ (ppm): 2.27 (3H, s, CH_3), 3.77 (3H, s, CH_3), 3.83 (3H, s, CH_3), 7.44 (1H, s, ind), 7.53 (1H, s, ind), 9.94 (1H, s, CHO).

General Procedure for the Synthesis of Compounds 3a−p. The appropriate 2-chloroaldehyde **1** (10 mmol) was dissolved in methanol (100 mL) and treated with the equivalent of the appropriate indolinone **2** and piperidine (1 mL). The reaction mixture was refluxed for 3−5 h (according to a TLC test), and the precipitate formed on cooling was collected by filtration and crystallized from ethanol with a yield of 10% for compound **3a**, 35−45% for compounds **3h**−**l**, **3n**, and **3p**, and 75−85% for compounds **3c**−**g**, **3m**, and **3o**.

Compound **3b** was prepared starting from 2-chloro-5-methoxy-1,6-dimethylindole-3-carbaldehyde (5 mmol), 2-indolinone (5 mmol), glycine (8 mmol), and sodium carbonate (3 mmol). The reaction mixture was refluxed for 24 h, and after cooling, the precipitate was collected by filtration and crystallized from toluene with a yield of 22%.

(b) In Vitro Growth Inhibition and Cytotoxicity. It was determined by the NCI according to standard procedures.[6]

(c) Effects on IGROV-1 Ovarian Carcinoma Cells. The ovarian carcinoma cell line IGROV-1 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mmol glutamine at 37 °C in air/5% CO_2. Compounds were dissolved in dimethylsulfoxide and diluted to the required concentration in complete medium. For treatments, cells were plated at 2×10^4 cells/cm^2 in a plastic well (60 cm^2), and after 24 h, the medium was removed and fresh medium containing the drugs was added. Control cells received the corresponding volume of dimethylsulfoxide. The number of viable cells was determined by trypan blue exclusion. The activity of caspase enzymes hydrolyzing the peptide sequence DEVD was measured in cell extracts by a fluorimetric assay.[1,15] One unit (U) of enzyme activity catalyzes the formation of 1 nmol of product per minute.

The rate of DNA synthesis was determined by thymidine incorporation. IGROV-1 cells were grown for 24 h and then treated with different drugs for an additional 24 h. Six hours before the end of incubation, 1 μCi/mL ^3H-thymidine was added to the culture medium; the cells were then washed in ice cold PBS and treated with 5% TCA for 30 min. They were then washed with PBS twice and incubated with 0.5 M NaOH at 50 °C for 60 min. The supernatant was collected and counted in a β-counter. Radioactivity was normalized for mg of proteins.

To determine cell cycle distribution, at the end of incubation, IGROV-1 cells were detached with 0.11% trypsin/0.02% EDTA, washed in PBS, and centrifuged. 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 flow cytometer Bryte (Biorad) and cell cycle analysis was performed using the M Cycle (Verity) and Modfit 5.0 software.

For immunofluorescence analysis of the tubulin polymerization level, IGROV-1 cells were seeded at 1×10^4 cells on a glass cover slip and, after 24 h, were treated with the compounds under study for 24 h. The cells were then washed with PBS, fixed with paraformaldehyde, and permeabilized in 70% ice-cold ethanol for 2 min at −20 °C. The samples were incubated with anti-α-tubulin, monoclonal antibody (Upstate) overnight at 4 °C and then incubated with antimouse conjugated with FITC (Sigma) for 1 h at room temperature. All preparations were embedded in Moviol and antibleaching DABCO and analyzed using a Nikon-Eclipse 90I microscope. Images were captured and analyzed using an ACP-2U software.

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Supporting Information Available: Analytical and spectroscopic data for all the new compounds. Tables related to COMPARE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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