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Substituted *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones with antitumor activity☆

Aldo Andreani,[a,*] Massimiliano Granaiola,[a] Alberto Leoni,[a] Alessandra Locatelli,[a] Rita Morigi,[a] Mirella Rambaldi,[a] Vida Garaliene,[b] Giovanna Farruggia[c] and Lanfranco Masotti[c]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[b]*Lithuanian Institute of Cardiology, Kaunas, LT-3007, Lithuania*
[c]*Dipartimento di Biochimica 'G. Moruzzi', Universita' di Bologna, Via Irnerio 48, 40126 Bologna, Italy*

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Abstract—The synthesis and antitumor activity of a new series of *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones is described. Several compounds were active on the primary test (three human cell lines) and entered the second level (60 human cell lines). All of them were potent growth inhibitors with GI_{50} ranging from −5.32 to −7.27. Four are now under review by BEC (Biological Evaluation Committee of the NCI). The most potent antitumor derivatives were also evaluated as cardiotonic agents (in view of a possible coanthracyclinic activity). In order to find a possible mechanism of action their effects on cell cycle progression in an adenocarcinoma cell line (HT29) were tested, evidencing that these molecules are able to block HT29 in mitosis. The introduction of new substituents in the indolinone moiety while maintaining the same chloroindole portion generated interesting derivatives. 3-(2-Chloro-5-methoxy-6-methyl-3-indolylmethylene)5-hydroxy-1,3-dihydroindol-2-one was the most active of the whole series. It was more potent than vincristine against seven of the nine tumors considered. Moreover it was selective towards some cell lines such as MDA-MB-435 (breast), OVCAR-3 (ovarian) and SK-MEL-28 (melanoma). Even the introduction of a benzyl ring at the nitrogen of the chloroindole portion, gave rise to potent compounds.

1. Introduction

We report the synthesis and antitumor activity of a new series of *E*-3-(2-chloro-3-indolylmethylene)1,3-dihydroindol-2-ones (**3**, see Scheme 1). It is our fourth paper on this topic.[2–4] Our previous paper[4] pointed out that all the most active compounds were derivatives of 5-methoxy-6-methyl-2-chloroindol-3-carbaldehyde **1** (R=H).

At the light of this finding we planned the synthesis of new compounds **3** according to the following rationale:

1. introduction of new substituents in the indolinone moiety (**3a**–**g**) while maintaining the same chloroindole portion,
2. introduction of an unsubstituted (**3h**–**n**) or substituted (**3o**–**q**) benzyl ring at the nitrogen of the chloroindole portion, while leaving the indolinone nitrogen as NH since in a previous paper[2] we demonstrated that at least one of the two nitrogens should be unsubstituted.

The growth inhibition of all the new compounds (**3a**–**q**) was tested on three human tumor cell lines according to the protocols developed at the National Cancer Institute (NCI, Bethesda, MD). The active compounds were tested on the sixty tumor cell lines.

The most potent antitumor derivatives (**3c**,**f**,**m**,**o**) were also evaluated in their effects on cell proliferation and cell cycle progression in the colon adenocarcinoma cell line HT29, because it has been demonstrated that several indole-derivatives are able to induce a G2/M arrest[5,6] and apoptosis in different cell lines.[7] Finally they were tested as positive inotropic agents in search for a potential coanthracyclinic activity. We suggested this term to indicate the pharmacological behavior of a molecule endowed with both antitumor activity

* Corresponding author. Tel.: +39-051-2099714; fax: +39-051-2099734; e-mail: aldo.andreani@unibo.it
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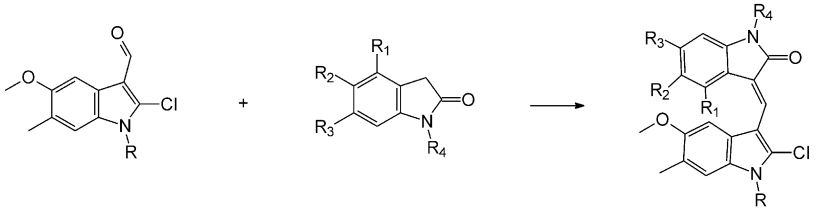

1 2 3

Scheme 1.

Table 1. Compounds **3a**–**q** (Bn = benzyl)

Comp	R	R_1	R_2	R_3	R_4	Formula	MW	Mp, °C
3a	H	Cl	H	H	H	$C_{19}H_{14}Cl_2N_2O_2$	373.2	335–337 dec.
3b	H	H	Cl	H	H	$C_{19}H_{14}Cl_2N_2O_2$	373.2	215–220 dec.
3c	H	H	OH	H	H	$C_{19}H_{15}ClN_2O_3$	354.8	285–287 dec.
3d	H	H	OC_2H_5	H	H	$C_{21}H_{19}ClN_2O_3$	382.8	204–206
3e	H	H	OH	CH_3	H	$C_{20}H_{17}ClN_2O_3$	368.8	300–302 dec.
3f	H	H	OH	H	CH_3	$C_{20}H_{17}ClN_2O_3$	368.8	340–345 dec.
3g	H	H	OCH_3	H	CH_3	$C_{21}H_{19}ClN_2O_3$	382.8	285–289 dec.
3h	Bn	H	H	H	H	$C_{26}H_{21}ClN_2O_2$	428.9	197–200
3i	Bn	H	OCH_3	H	H	$C_{27}H_{23}ClN_2O_3$	458.9	189–190
3j	Bn	H	OCH_3	CH_3	H	$C_{28}H_{25}ClN_2O_3$	473.0	211–214 dec.
3k	Bn	Cl	H	H	H	$C_{26}H_{20}Cl_2N_2O_2$	463.4	270–272 dec.
3l	Bn	H	Cl	H	H	$C_{26}H_{20}Cl_2N_2O_2$	463.4	190–192 dec.
3m	Bn	H	OH	H	H	$C_{26}H_{21}ClN_2O_3$	444.9	200–205 dec.
3n	Bn	H	OH	CH_3	H	$C_{27}H_{23}ClN_2O_3$	458.9	293–295 dec.
3o	4Cl-Bn	H	H	H	H	$C_{26}H_{20}Cl_2N_2O_2$	463.4	215–217 dec.
3p	$4CH_3O$-Bn	H	H	H	H	$C_{27}H_{23}ClN_2O_3$	458.9	258–260 dec.
3q	$2NO_2$-Bn	H	H	H	H	$C_{26}H_{20}ClN_3O_4$	473.9	221–223 dec.

(in order to reduce the anthracycline toxicity by reducing its dosage) and positive inotropic activity (in order to counteract the heart depression induced by anthracyclines).[8]

2. 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 for compound **3f** triethylamine was employed since piperidine gave nucleophilic displacement of chlorine at the 2 position with the formation of the 2-piperidinyl derivative. This behavior was previously noticed for analogue compounds.[2,4]

The NMR spectra are in agreement with those previously published for analogue *E*-derivatives and are reported in Table 2. A common feature in this series is the hydrogen at position 4 in the 2-chloroaldehydes (7.5–7.6 ppm in compounds **1**) which is shielded by the indolinone portion (6.5–6.9 ppm for the same hydrogen in compounds **3**).

All compounds **3** were obtained as pure geometrical isomers. In order to confirm that they belong (as the previous ones) to the *E* configuration, the usual NOE experiments were performed on compounds **3e**,**h**,**q**.

3. Pharmacological results

3.1. In vitro growth inhibition and cytotoxicity[9]

As a primary screening, compounds **3a**–**q** were evaluated for their cytotoxic potency on 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 (negative numbers indicate cell kill). Eleven 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 organised 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 and the others 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M.

Table 3 reports the results obtained expressed as $\log_{10}$ of the molar concentration, taking into consideration the 50% growth inhibitory power (GI_{50}), the cytostatic effect (TGI = Total Growth Inhibition) and the cytotoxic effect (LC_{50}).

The four most active compounds (**3c**,**f**,**m**,**o**) have been analyzed by means of COMPARE.[10] This program

Table 2. IR and ^{1}H NMR of compounds **3a–q**

Comp	IR: ν_{max} cm^{-1}	^{1}H NMR:[a]δ, ppm in DMSO-d_6
3a	1665, 1614, 1209, 717	2.25 (3H, s, CH_3), 3.75 (3H, s, OCH_3), 6.83 (1H, dd, ox, $J=7.9$, $J=1$), 6.85 (1H, s, ind-4), 7.01 (1H, dd, ox, $J=7.9$, $J=1$), 7.15 (1H, s, ind-7), 7.19 (1H, t, ox, $J=7.9$), 8.42 (1H, s, CH), 10.77 (1H, s, NH-ox), 12.54 (1H, s, NH-ind)
3b	1665, 1591, 1301, 1204	2.28 (3H, s, CH_3), 3.61 (3H, s, OCH_3), 6.49 (1H, s, ind-4), 6.77 (1H, d, ox-4, $J=2.2$), 6.90 (1H, d, ox-7, $J=8.2$), 7.24 (1H, dd, ox-6, $J=8.2$, $J=2.2$), 7.27 (1H, s, ind-7), 7.73 (1H, s, CH), 10.72 (1H, s, NH-ox), 12.74 (1H, s, NH-ind)
3c	1679, 1596, 1199, 1132	2.27 (3H, s, CH_3), 3.61 (3H, s, OCH_3), 6.38 (1H, d, ox-4, $J=2.1$), 6.56 (1H, s, ind-4), 6.60 (1H, dd, ox-6, $J=8.4$, $J=2.1$), 6.67 (1H, d, ox-7, $J=8.4$), 7.23 (1H, s, ind-7), 7.58 (1H, s, CH), 8.85 (1H, s, OH), 10.27 (1H, s, NH-ox), 12.56 (1H, s, NH-ind)
3d	1664, 1593, 1281, 1189	1.13 (3H, t, CH_3CH_2, $J=7$), 2.26 (3H, s, CH_3), 3.58 (3H, s, OCH_3), 3.71 (2H, q, CH_3CH_2, $J=7$), 6.41 (1H, s, ox-4), 6.54 (1H, s, ind-4), 6.77 (2H, s, ox-6,7), 7.24 (1H, s, ind-7), 7.64 (1H, s, CH), 10.37 (1H, s, NH-ox), 12.50 (1H, broad, NH-ind)
3e	1670, 1598, 1322, 1183	2.10 (3H, s, CH_3), 2.26 (3H, s, CH_3), 3.62 (3H, s, OCH_3), 6.47 (1H, s, ox-4), 6.57 (1H, s, ox-7), 6.62 (1H, s, ind-4), 7.21 (1H, s, ind-7), 7.50 (1H, s, CH), 8.67 (1H, s, OH), 10.19 (1H, s, NH-ox), 12.46 (1H, s, NH-ind)
3f	1664, 1588, 1209, 1143	2.27 (3H, s, CH_3), 3.19 (3H, s, N-CH_3), 3.60 (3H, s, OCH_3), 6.43 (1H, d, ox-4, $J=2.5$), 6.55 (1H, s, ind-4), 6.68 (1H, dd, ox-6, $J=8.4$, $J=2.5$), 6.83 (1H, d, ox-7, $J=8.4$), 7.24 (1H, s, ind-7), 7.67 (1H, s, CH), 8.96 (1H, s, OH), 12.59 (1H, broad, NH)
3g	1685, 1590, 1289, 723	2.26 (3H, s, CH_3), 3.22 (3H, s, N-CH_3), 3.49 (3H, s, OCH_3), 3.55 (3H, s, OCH_3), 6.46 (1H, d, ox-4, $J=2.3$), 6.51 (1H, s, ind-4), 6.88 (1H, dd, ox-6, $J=8.5$, $J=2.3$), 6.95 (1H, d, ox-7, $J=8.5$), 7.25 (1H, s, ind-7), 7.73 (1H, s, CH), 12.62 (1H, broad, NH)
3h	1716, 1609, 1245, 1045	2.25 (3H, s, CH_3), 3.56 (3H, s, OCH_3), 5.58 (2H, s, CH_2), 6.60 (1H, s, ind-4), 6.81 (2H, m, ox-4,5), 6.89 (1H, d, ox-7, $J=7.6$), 7.15 (2H, d, ph, $J=6.8$), 7.19 (1H, m, ox-6), 7.31 (3H, m, ph), 7.52 (1H, s, ind-7), 7.67 (1H, s, CH), 10.63 (1H, s, NH)
3i	1711, 1619, 1204, 1035	2.25 (3H, s, CH_3), 3.40 (3H, s, OCH_3), 3.60 (3H, s, OCH_3), 5.58 (2H, s, CH_2), 6.41 (1H, s, ox-4), 6.65 (1H, s, ind-4), 6.79 (2H, s, ox-6,7), 7.15 (2H, d, ph, $J=6.9$), 7.30 (3H, m, ph), 7.52 (1H, s, ind-7), 7.67 (1H, s, CH), 10.44 (1H, s, NH)
3j	1685, 1593, 1260, 1091	2.12 (3H, s, CH_3), 2.25 (3H, s, CH_3), 3.18 (3H, s, OCH_3), 3.62 (3H, s, OCH_3), 5.57 (2H, s, CH_2), 6.38 (1H, s, ox-4), 6.68 (1H, s, ind-4), 6.72 (1H, s, ox-7), 7.14 (2H, d, ph, $J=6.7$), 7.31 (3H, m, ph), 7.51 (1H, s, ind-7), 7.60 (1H, s, CH), 10.35 (1H, s, NH)
3k	1696, 1655, 1593, 697	2.24 (3H, s, CH_3), 3.76 (3H, s, OCH_3), 5.55 (2H, s, CH_2), 6.84 (1H, d, ox, $J=7.8$), 6.90 (1H, s, ind-4), 7.02 (1H, d, ox, $J=7.8$), 7.16 (2H, d, ph, $J=6.3$), 7.20 (1H, t, ox-6, $J=7.8$), 7.31 (3H, m, ph), 7.40 (1H, s, ind-7), 8.44 (1H, s, CH), 10.80 (1H, s, NH)
3l	1706, 1609, 1301, 697	2.26 (3H, s, CH_3), 3.64 (3H, s, OCH_3), 5.59 (2H, s, CH_2), 6.62 (1H, s, ind-4), 6.78 (1H, d, ox-4, $J=2.1$), 6.90 (1H, d, ox-7, $J=8.5$), 7.18 (2H, d, ph, $J=7.3$), 7.25 (1H, dd, ox-6, $J=8.5$, $J=2.1$), 7.34 (3H, m, ph), 7.54 (1H, s, ind-7), 7.77 (1H, s, CH), 10.77 (1H, s, NH)
3m	1675, 1606, 1199, 692	2.26 (3H, s, CH_3), 3.61 (3H, s, OCH_3), 5.58 (2H, s, CH_2), 6.39 (1H, d, ox-4, $J=2.1$), 6.62 (1H, dd, ox-6, $J=8$, $J=2.1$), 6.62 (1H, s, ind-4), 6.69 (1H, d, ox-7, $J=8$), 7.17 (2H, d, ph, $J=7.3$), 7.32 (3H, m, ph), 7.50 (1H, s, ind-7), 7.62 (1H, s, CH), 8.87 (1H, s, OH), 10.29 (1H, s, NH)
3n	1670, 1598, 1322, 1183	2.11 (3H, s, CH_3), 2.26 (3H, s, CH_3), 3.61 (3H, s, OCH_3), 5.58 (2H, s, CH_2), 6.47 (1H, s, ox-4), 6.60 (1H, s, ox-7), 6.66 (1H, s, ind-4), 7.17 (2H, d, ph, $J=7.2$), 7.31 (3H, m, ph), 7.49 (1H, s, ind-7), 7.54 (1H, s, CH), 8.67 (1H, s, OH), 10.23 (1H, s, NH)
3o	1711, 1609, 1245, 738	2.25 (3H, s, CH_3), 3.57 (3H, s, OCH_3), 5.58 (2H, s, CH_2), 6.61 (1H, s, ind-4), 6.84 (3H, m, ox), 7.16 (2H, d, ph, $J=8.5$), 7.17 (1H, m, ox), 7.41 (2H, d, ph, $J=8.5$), 7.52 (1H, s, ind-7), 7.66 (1H, s, CH), 10.62 (1H, s, NH)
3p	1706, 1609, 1511, 1240	2.26 (3H, s, CH_3), 3.56 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 5.49 (2H, s, CH_2), 6.58 (1H, s, ind-4), 6.84 (3H, m, ox), 6.90 (2H, d, ph, $J=8.6$), 7.14 (2H, d, ph, $J=8.6$), 7.21 (1H, m, ox), 7.53 (1H, s, ind-7), 7.67 (1H, s, CH), 10.61 (1H, s, NH)
3q	1698, 1606, 1524, 1245	2.20 (3H, s, CH_3), 3.60 (3H, s, OCH_3), 5.97 (2H, s, CH_2), 6.35 (1H, m, ph-6), 6.68 (1H, s, ind-4), 6.90 (3H, m, ox), 7.21 (1H, m, ox), 7.51 (1H, s, ind-7), 7.60 (2H, m, ph-4,5) 7.70 (1H, s, CH), 8.25 (1H, m, ph-3), 10.63 (1H, s, NH)

The NH and OH groups give broad bands in the range 3270–3030 cm^{-1}.

[a] Abbreviations: ind = chloroindole, ox = oxindole, ph = phenyl.

compared the patterns evinced by the test compounds with the patterns produced by the standard anticancer agents in the NCI database.[11] Among these drugs we chose vincristine since its structure contains two indoles and we reported in Table 3 the available data for comparison purposes (the highest dose tested was 10^{-3} instead of 10^{-4}).

All the compounds were potent growth inhibitors with GI_{50} ranging from −4.90 to −7.27. Four of them (**3c**,**f**,**m**,**o**) are now under review by BEC (Biological Evaluation Committee of the NCI).

3.2. Growth inhibition and cell cycle arrest in colon adenocarcinoma HT29 cell line

In order to investigate the mechanism of action of these drugs, the antiproliferative effects of the four most potent antitumor agents (**3c**,**f**,**m**,**o**) were studied by monitoring their effects on cell proliferation and cell cycle in a colon adenocarcinoma cell line, HT29. This line was chosen because all these compounds demonstrated a high activity on colon cancer cell lines as shown in Table 3. The effects of treatments in the range of concentrations between 10^{-7} and 10^{-5} are shown in Figure 1. The highest dose (10^{-4}M) of all these molecules caused a marked cytotoxic effect, with the death of the whole culture (data not shown). It was observed that all the compounds at the doses 10^{-5} and 10^{-6}M induced a decrease of cell growth after 24 (Fig. 1A) and 48 h (Fig. 1B) of treatment, but compounds **3c** and **3f** were more effective and were able to decrease cell proliferation at lower doses than compound **3m** and **3o**. Furthermore compound **3m** at the lower doses did not show any effect, and after 48 h seemed to stimulate cell growth. In order to clarify this proliferative arrest in more detail the analysis of DNA profiles was performed using propidium iodide staining and flow cytometry, and Figure 2 shows the profile of DNA of the cells treated with **3c** for 24 and 48 h. In Figure 3 the percentage of cell in G2/M phase obtained with all the treatments are reported. All the four compounds were able to induce a block in G2/M phase, with different efficiency: after 24 h (Fig. 3A) of 10^{-6}M **3c** treatment, more than 90% of the cells were in G2/M phase, while the percentages of cells in this cell cycle phase for compounds **3f**, **3m** and **3o** were respectively around 70%, 7% and 55% confirming the lower potency of these compounds and the lack of activity of **3m** at this concentration. However the 10^{-5} M dose was able to induce over than 80% of the cells to arrest in the G2/M phase with all the tested compounds. 48 h of treatments

Table 3. Sixty cell panel (growth inhibition, cytostatic and cytotoxic activity of the selected compounds)

NSC	Comp [a]	Modes	Leukemia	NSCLC	Colon	CNS	Melanoma	Ovarian	Renal	Prostate	Breast	MG−MID[b]
723558	**3a**	GI_{50}	−5.88	−5.61	−5.73	−5.71	−5.66	−5.89	−5.63	−5.65	−5.80	−5.73
		TGI	−5.50	−5.08	−5.39	−5.25	−5.15	−5.13	−5.12	−5.09	−5.35	−5.23
		LC_{50}	−5.08	−4.42	−5.03	−4.75	−4.66	−4.49	−4.62	−4.56	−4.74	−4.71
723557	**3b**	GI_{50}	−6.40	−5.88	−6.29	−6.42	−6.23	−6.13	−6.06	−6.08	−6.27	−6.19
		TGI	−5.58	−4.89	−5.12	−5.17	−4.91	−5.03	−4.92	−5.15	−5.21	−5.09
		LC_{50}	−4.50	−4.12	−4.33	−4.35	−4.30	−4.22	−4.34	−4.60	−4.22	−4.30
723559	**3c**	GI_{50}	−7.22	−6.38	−7.23	−7.12	−7.27	−7.03	−6.78	−6.66	−7.24	−6.99
		TGI	−5.17	−5.15	−5.79	−5.29	−5.46	−5.86	−5.15	−5.10	−5.66	−5.42
		LC_{50}	−4.08	−4.22	−4.34	−4.31	−4.43	−4.28	−4.34	−4.40	−4.66	−4.34
723560	**3e**	GI_{50}	−6.18	−5.49	−5.61	−5.56	−5.57	−5.53	−5.49	−5.53	−5.66	−5.62
		TGI	−5.54	−4.82	−5.07	−4.96	−4.83	−4.91	−4.95	−4.82	−4.99	−4.99
		LC_{50}	−4.67	−4.27	−4.53	−4.39	−4.36	−4.37	−4.28	−4.24	−4.29	−4.38
723554	**3f**	GI_{50}	−6.60	−6.10	−6.45	−6.39	−6.49	−6.26	−6.23	−5.83	−6.49	−6.34
		TGI	−5.13	−4.99	−5.37	−5.56	−5.12	−5.28	−4.91	−4.71	−5.48	−5.18
		LC_{50}	−4.11	−4.30	−4.46	−4.34	−4.32	−4.38	−4.32	−4.19	−4.51	−4.34
723565	**3k**	GI_{50}	−5.18	−5.22	−5.61	−5.56	−5.46	−5.02	−5.49	−4.96	−5.44	−5.36
		TGI	–	−4.31	−4.47	−4.30	−4.57	−4.07	−4.31	–	−4.53	−4.33
		LC_{50}	–	–	–	−4.02	−4.05	−4.02	–	–	–	−4.01
723566	**3l**	GI_{50}	−5.54	−5.52	−5.39	−5.52	−5.44	−5.64	−5.61	−5.32	−5.41	−5.50
		TGI	−4.94	−4.77	−4.53	−4.93	−4.68	−5.21	−5.22	−4.72	−4.56	−4.84
		LC_{50}	−4.12	−4.15	−4.01	−4.26	−4.18	−4.18	−4.42	−4.14	−4.18	−4.19
723564	**3m**	GI_{50}	−6.28	−5.98	−6.41	−6.37	−6.18	−6.18	−6.03	−5.97	−6.23	−6.19
		TGI	−4.45	−4.66	−5.43	−5.09	−4.62	−4.88	−4.49	−4.29	−5.22	−4.80
		LC_{50}	–	−4.15	−4.34	−4.19	−4.11	−4.37	−4.07	–	−4.24	−4.17
723568	**3o**	GI_{50}	−6.62	−6.23	−6.51	−6.49	−6.38	−6.45	−6.43	−6.46	−6.48	−6.44
		TGI	−6.20	−5.53	−5.84	−5.59	−5.28	−5.40	−5.63	−5.44	−5.48	−5.60
		LC_{50}	−5.28	−4.47	−4.65	−4.32	−4.39	−4.71	−4.53	−4.54	−4.67	−4.61
723570	**3p**	GI_{50}	−5.33	−5.05	−5.45	−5.31	−5.12	−5.30	−5.19	−4.97	−5.28	−5.22
		TGI	−4.02	−4.12	−4.38	−4.12	−4.29	−4.21	−4.07	–	−4.36	−4.20
723569	**3q**	GI_{50}	−5.32	−5.05	−5.39	−4.90	−5.16	−5.04	−5.06	−5.23	−5.25	−5.14
		TGI	−4.04	−4.40	−4.64	−4.43	−4.58	−4.40	−4.48	−4.29	−4.46	−4.44
		LC_{50}	–	−4.05	−4.04	−4.11	−4.14	−4.09	−4.10	–	−4.07	−4.08
67574	Vincristine sulfate [c]	GI_{50}	−7.00	−6.60	−7.00	−6.90	−6.80	−6.50	−6.50	−6.90	−6.50	−6.70
		TGI	−4.80	−4.80	−5.40	−5.20	−5.10	−4.70	−4.70	−5.20	−5.10	−5.00
		LC_{50}	−3.20	−3.60	−4.10	−3.70	−3.60	−3.50	−3.60	−3.50	−3.50	−3.60

[a] All values are $\log_{10}$. Highest conc. = 10^{-4}M; only modes showing value > −4.00 are reported.
[b] Calculated mean panel.
[c] Highest conc. = 10^{-3}M.

(Fig. 3B) did not significantly modify this distribution, indicating that for **3c**,**f**,**o** it was sufficient to incubate cells in presence of a concentration of 10^{-6} M to achieve a block in the cell cycle progression, whereas compound **3m** needed a 10-fold increase in concentration to show its synchronizing effects. The analysis of DNA profiles did not show an appreciable presence of nuclei with fragmented DNA, suggesting that these compounds induce G2/M arrest but not apoptosis.

To determine whether cells were arrested in G2 or in mitosis after treatments, chromatin was visualized by Hoechst 33432 staining. Results showed that after 24 h of treatments with all the four compounds, a high percentage of cells had condensed chromosomes and the mitotic block increased after 48 h. The same results were obtained with the dose 10^{-6} M of compound **3c**,**f**,**o**. An example of these results is shown in Figure 4, where the photomicrographs of the control and of the cells treated for 24 and 48 h with **3c** 10^{-5} M are reported. Furthermore microscopic evaluation confirmed that a significant increase of apoptotic cells in treated samples did not occur.

3.3. Positive inotropic activity

The positive inotropic activity of the most potent antitumor agents (**3c**,**f**,**m**,**o**) was evaluated in view of a possible coanthracyclinic[8] activity. With the procedure described, compound **3m** showed a weak but significant

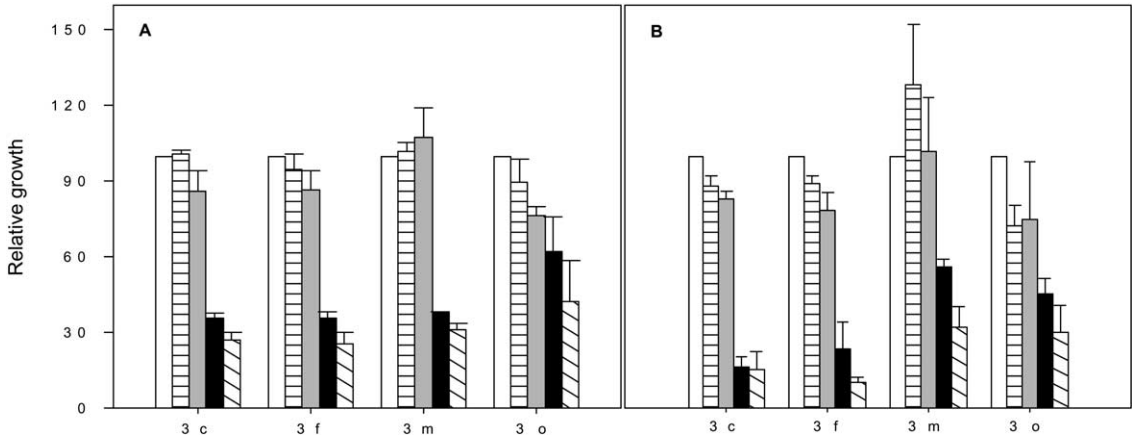

Figure 1. Relative cell growth after 24 h (A) and 48 h (B) treatment, calculated respect to control, taken as 100%. control cells, compounds **3c, 3f, 3m, 3o** 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M.

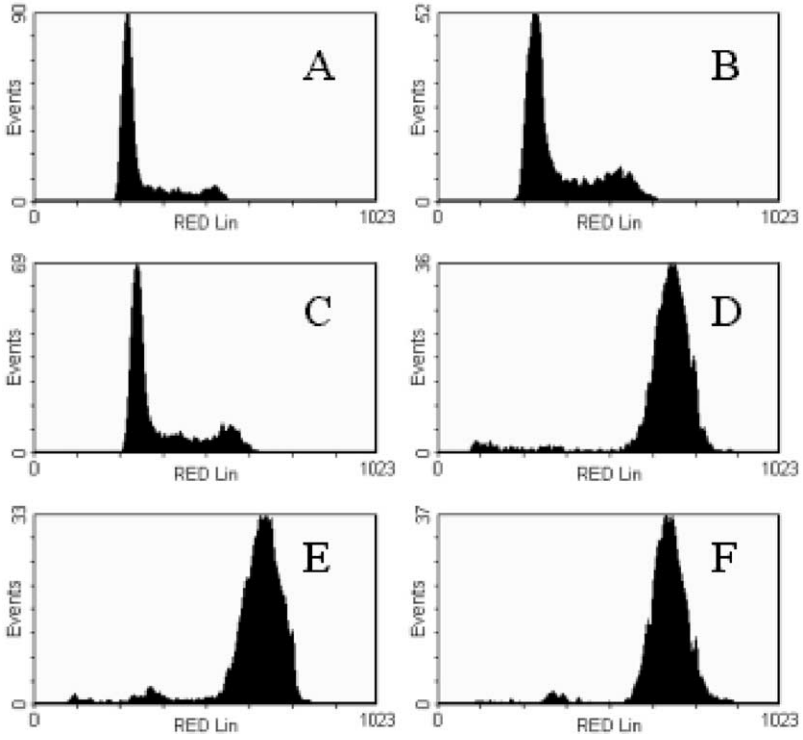

Figure 2. DNA profiles of cells treated for 24 h with compound **3c**: A, control cells; B, **3c** 10^{-8} M; C, **3c** 10^{-7} M; D, **3c** 10^{-6} M; E, **3c** 10^{-5} M. F, cell treated with **3c** 10^{-5} M for 48 h.

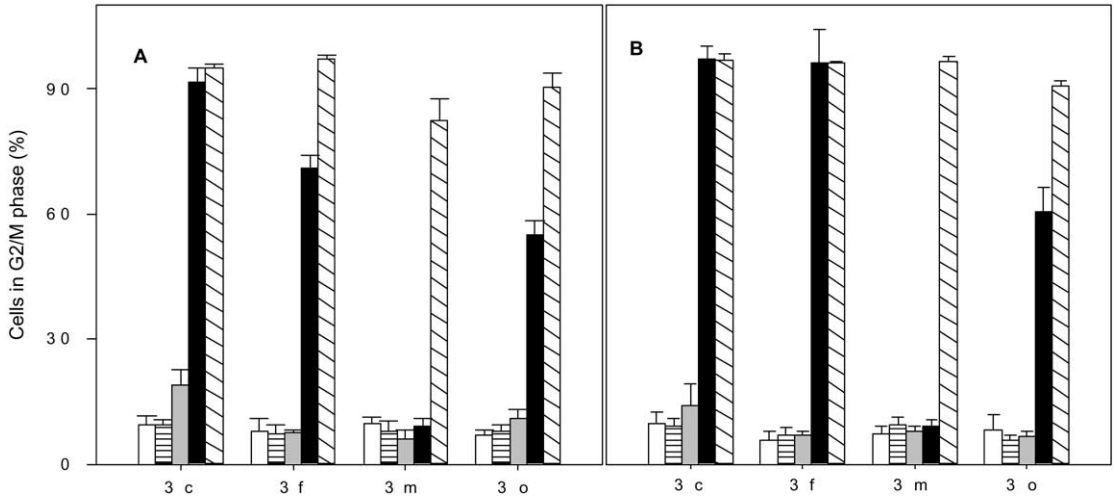

Figure 3. Percentage of cell in G2/M phase after 24 h (A) and 48 h (B) of treatments. control cells, compounds **3c, 3f, 3m, 3o** 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5} M. The distribution of HT29 cells within the cell cycle was analyzed by flow cytometry as described in Section 5.

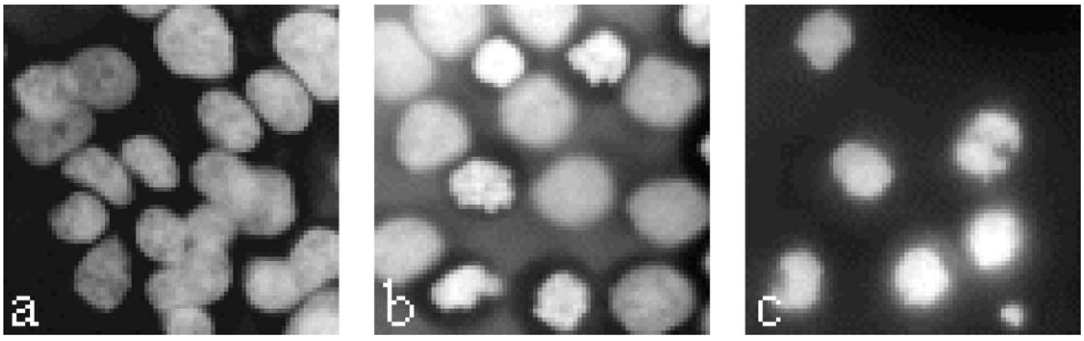

Figure 4. Photographs of control cells (a), cells treated with **3c** 10^{-5} M for 24 h (b) and 48 h (c). Cells were stained by Hoechst 33432 as reported in Section 5.

cardiotonic activity at 10^{-7}M. The well known positive inotropic agent amrinone, at the same concentration, did not produce any effect. In our opinion compound **3m** could be a promising candidate for testing its effect on the antitumor/cardiotoxic activity of anthracyclines. Unfortunately, as far as we know, in this moment there are no laboratories performing this test.

4. Conclusions

The first approach (introduction of new substituents in the indolinone moiety while maintaining the same chloroindole portion) has been successful since generated interesting derivatives such as **3a**–**c**,**f**. Compounds **3a**, bearing a chlorine at position 4, was less active than the 5-chloro analogue (**3b**) but was selective towards the ovarian cancer OVCAR-4 (GI_{50} −7.11). Compounds **3c** (bearing a OH group at the 5 position) and **3f** (which, compared to **3c**, bears a methyl group in position 1) were selected by BEC for a possible further development. Compound **3c** was the most active of the whole series. It was more potent than vincristine against seven of the nine tumors considered. Moreover it was selective towards some cell lines such as MDA-MB-435 (breast), OVCAR-3 (ovarian) and SK-MEL-28 (melanoma).

Even the second approach, concerning also the introduction of a benzyl ring at the nitrogen of the chloroindole portion, gave rise to potent compounds selected by BEC (**3m** and **3o**).

This study points out that the substituent at the 5 position of the indolinone portion is much more critical than the substituents considered in all the other positions and from the results up to now obtained it seems that an hydrogen[4] or a small substituent represents the best solution.

Flow cytometric analysis and microscopic evaluation of the effects on cell cycle progression induced by compounds **3c**,**f**,**m**,**o** showed that these molecules are able to arrest adenocarcinoma HT29 cells in mitosis, even if with different potency. The most active compound was **3c**, whereas the presence of a benzyl ring at the nitrogen of the chloroindole portion decreased this activity. The arrest in mitosis suggest that these compounds could act by inhibiting tubulin polymerization as other bis-indole derivatives and inducing the so called 'mitotic catastrophe'.[6] They demonstrated to be more active than vincristine, which is not able to completely synchronize several cell lines in G2/M phase.[5] Furthermore, vincristine induces apoptosis in several tumor lines, including

colon carcinoma in vivo and in vitro,[12] but as far as we know its behavior on HT29 cells has never been reported in the literature.

5. Experimental

5.1. 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. Kieselgel 60 (Merck) was used for column chromatography. 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. (Table 2). 2-Indolinone is commercially available whereas the substituted 2-indolinones[4,13–16] and 2-chloro-5-methoxy-6-methylindole-3-carbaldehyde[17] were prepared according to the literature. The synthesis of four new aldehydes is reported below.

5.2. General procedure for the synthesis of the four new aldeydes

2-chloro-5-methoxy-6-methylindole-3-carbaldehyde (10 mmol) was dissolved in DMF (10 mL) and treated portionwise, under stirring, with NaH (15 mmol). The reaction mixture was stirred at room temperature for 10 min, treated with the appropriate benzyl chloride (60 mmol) and maintained at 100 °C for 1–3 h (according to a TLC test). The reaction mixture was poured onto ice and the resulting precipitate was recovered by filtration with a yield of 70–80%. It was then purified by crystallization with ethanol.

5.3. 1-Benzyl-2-chloro-5-methoxy-6-methylindole-3-carbaldehyde (1, R=Bn)

$C_{18}H_{16}ClNO_2$ (313.8). Mp 139–140 °C ν_{max} cm^{-1}: 1660, 1511, 1255, 1045 δ, ppm: 2.22 (3H, s, CH_3), 3.83 (3H, s, OCH_3), 5.55 (2H, s, CH_2), 7.15 (2H, d, ph, $J=7.6$), 7.32 (3H, m, ph), 7.48 (1H, s, ind-7), 7.58 (1H, s, ind-4), 10.00 (1H, s, CHO).

5.4. 2-Chloro-1-(4-chlorobenzyl)-5-methoxy-6-methylindole-3-carbaldehyde (1, R=4Cl-Bn)

$C_{18}H_{15}Cl_2NO_2$ (348.2). Mp 176–179 °C dec. ν_{max} cm^{-1}: 1640, 1500, 1035, 715 δ, ppm: 2.23 (3H, s, CH_3), 3.84 (3H, s, OCH_3), 5.57 (2H, s, CH_2), 7.16 (2H, d, ph, $J=6.6$), 7.42 (2H, d, ph, $J=6.6$), 7.49 (1H, s, ind-7), 7.59 (1H, s, ind-4), 10.00 (1H, s, CHO).

5.5. 2-Chloro-5-methoxy-1-(4-methoxybenzyl)-6-methylindole-3-carbaldehyde (1, R=4CH$_3$O-Bn)

$C_{19}H_{18}ClNO_3$ (343.8). Mp 133-135 °C ν_{max} cm^{-1}: 1650, 1510, 1250, 1030 δ, ppm: 2.20 (3H, s, CH_3), 3.67 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 5.43 (2H, s, CH_2), 6.86 (2H, d, ph, $J=8.4$), 7.11 (2H, d, ph, $J=8.4$), 7.47 (1H, s, ind-7), 7.54 (1H, s, ind-4), 9.96 (1H, s, CHO).

5.6. 2-Chloro-5-methoxy-6-methyl-1-(2-nitro-benzyl)-indole-3-carbaldehyde (1, R=2NO$_2$Bn)

$C_{18}H_{15}ClN_2O_4$ (358.8). Mp 220–222 °C ν_{max} cm^{-1}: 1659, 1524, 1258, 1047 δ, ppm: 2.17 (3H, s, CH_3), 3.85 (3H, s, OCH_3), 5.93 (2H, s, CH_2), 6.34 (1H, m, ph-6), 7.48 (1H, s, ind-7), 7.59 (2H, m, ph-4,5), 7.62 (1H, s, ind-4), 8.26 (1H, m, ph-3), 10.04 (1H, s, CHO).

5.7. General procedure for the synthesis of compounds 3a–q

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). Only for compound **3f** the same amount of triethylamine was employed instead of piperidine. The reaction mixture was refluxed for 3–5 h (according to a TLC test) and the precipitate formed on cooling was collected by filtration. Compounds **3a**–**b** were purified by column chromatography and all the others by crystallization from ethanol or toluene. The yield was 20–30% for compounds **3a**–**f**,**m**, 50–60% for compounds **3g**,**n**,**q** and 70–80% for compounds **3h**–**l**,**o**–**p**.

5.8. In vitro growth inhibition and cytotoxicity

It was determined by the NCI according to standard procedures.[9]

5.9. Cell cycle analysis

HT29 cells (Istituto Zooprofilattico di Brescia, Italy) were routinely cultured in RPMI 1640 medium (Gibco,UK) supplemented by 10% heat-inactivated fetal calf serum (Euroclone, UK) and L-glutamine (Sigma, USA) 2 mmol, at 37 °C in 5% CO_2 atmosphere. Compounds under test (**3c**,**f**,**m**,**o**) were dissolved in DMSO (Sigma, USA) at 10 mg/mL immediately before use, and the different drugs concentrations were prepared in complete medium. In control cells only DMSO was added to the fresh culture medium. For treatments, cells were seeded in tissue culture plates (Orange, Belgium) at 20×10^3 cell/cm^2 and after 24 h the medium was removed and fresh medium containing the drugs at concentrations ranging between 10^{-4} and 10^{-8}M was added. After 24 and 48 h cells were trypsinized and counted; dead cells were stained by diluting cell samples 1:1 with 5% tripan blue solution. Growth was plotted as percentage of the control.

At the indicated time points, nuclei were isolated according to Nusse et al.[18] Isolated nuclei were kept at 4 °C in the dark. At the moment of the analysis, cells were stained with propidium iodide (Sigma, USA) 50 μg/mL. Cell cycle profiles were determined using an Epics Elite (Beckman Coulter, USA) and analyzed by Modfit software (Verity).

For microscopical evaluation, cells were grown on glass coverslip, washed 3 times in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Sigma, USA) in PBS and permeabilized with 0.5% Triton X-100 (Sigma, USA) in PBS for 5 min at 4 °C. After 2 washes in PBS, sample were incubated in presence of Hoechst 33432 (Sigma USA) 0,1 μg/mL, washed and analyzed by fluorescence microscopy.

5.10. Positive inotropic activity

The experiments were carried out on the guinea-pig papillary muscles according to a procedure previously described.[8]

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References and notes

1. Potential Antitumor Agents 35. For part 34 see Andreani, A.; Granaiola, M.; Leoni, A.; Locatelli, A.; Morigi, R.; Rambaldi, M.; Giorgi, G.; Garaliene, V. *Anticancer Res.* **2004**, *24*, 203.
2. Andreani, A.; Locatelli, A.; Rambaldi, M.; Leoni, A.; Bossa, R.; Fraccari, A.; Galatulas, I. *Anticancer Res.* **1996**, *16*, 3585.
3. Andreani, A.; Locatelli, A.; Leoni, A.; Morigi, R.; Chiericozzi, M.; Fraccari, A.; Galatulas, I.; Salvatore, G. *Eur. J. Med. Chem.* **1998**, *33*, 905.
4. Andreani, A.; Granaiola, M.; Leoni, A.; Locatelli, A.; Morigi, R.; Rambaldi, M.; Garaliene, V. *J. Med. Chem.* **2002**, *45*, 2666.
5. Comin-Anduix, B.; Agell, N.; Bachs, O.; Ovadi, J.; Cascante, M. *Mol. Pharm.* **2001**, *60*, 1235.
6. Mahboobi, S.; Pongratz, H.; Hufsky, H.; Hockmeyer, J.; Frieser, M.; Lyssenko, A.; Paper, D. H.; Burgermeister, J.; Bohmer, F.-D.; Fiebig, H.-H.; Burger, A. M.; Baasner, S.; Beckers, T. *J. Med. Chem.* **2001**, *44*, 4535.
7. Fan, M.; Goodwin, M. E.; Birrer, M. J.; Chambers, T. C. *Cancer Res.* **2001**, *61*, 4450.
8. Andreani, A.; Leoni, A.; Locatelli, A.; Morigi, R.; Rambaldi, M.; Recanatini, M.; Garaliene, V. *Bioorg. Med. Chem.* **2000**, *8*, 2359.
9. Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A. *J. Natl. Cancer Inst.* **1991**, *83*, 757.
10. http://dtp.nci.nih.gov/docs/compare/compare_methodology.html
11. http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent_table.html
12. Hannun, Y. A. *Blood* **1997**, *89*, 1845.
13. Koelsch, C. F. *J.Amer. Chem. Soc.* **1944**, *66*, 2019.
14. Romeo, A.; Corrodi, H.; Hardegger, E. *Helv. Chim. Acta* **1955**, *38*, 463.
15. Andreani, A.; Rambaldi, M.; Bonazzi, D.; Greci, L.; Andreani, F. *Farmaco, Ed. Sci.* **1979**, *34*, 132.
16. Beer, R. J. S.; Davenport, H. F.; Robertson, A. *J. Chem. Soc.* **1953**, 1262.
17. Porter, J. C.; Robinson, R.; Wyler, M. *J. Chem. Soc.* **1941**, 620.
18. Nusse, M.; Beisker, W.; Hoffman, C.; Tarnok, A. *Cytometry* **1990**, *11*, 813.