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# 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[1]

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The synthesis of new substituted *E*-3-(3-indolylmethylene)-1,3-dihydroindol-2-ones is reported. The antitumor activity was evaluated according to protocols available at the National Cancer Institute (NCI), Bethesda, MD. Structure−activity relationships are discussed. The action of selected compounds was investigated in MCF-7 breast cancer cells. The ability of these derivatives to inhibit cellular proliferation was accompanied by increased level of p53 and its transcriptional targets p21 and Bax, interference in the cell cycle progression with cell accumulation in the G2/M phase, and activation of apoptosis.

## Introduction

In the previous papers of this series[2−6] we described several substituted *E*-3-(2-chloro-3-indolylmethylene)-1,3-dihydroindol-2-ones prepared from an indolealdehyde and an oxindole. Compounds with potent cytotoxic activity were obtained when the aldehyde was 2-chloro-5-methoxy-6-methylindole-3-carbaldehyde.[4] Moreover, we noticed[6] that in some cases N-methylation increased the activity. Even preliminary attempts to introduce substituents at positions 4, 5, and 6 of the oxindole portion had been performed.[4]

The rationale for the synthesis of new analogues described in this paper (Scheme 1, Table 1) is the following:

1. Evaluation of the effects induced by different substituents in the oxindole portion (bromine, dimethylamino, and succinyl groups **4**, **5**, and **6**) and by the position of chlorine (**7**) was done: the compounds bearing chlorine at the position 4 or 5 were described in previous papers.[5,6]
2. Compound **8** was prepared to evaluate whether the methylation of the nitrogen in the chloroindole portion could lead to increase of activity as previously noticed.[5,6] This kind of methylation was also performed in four compounds mentioned in the above group (**4**−**7**) and gave rise to the derivatives **9**−**12**.
3. The synthesis of one compound bearing a methyl group at both nitrogens and its activity on HeLa cells was described in the first paper of this series.[2] Its cytotoxicity was weak, but when it was tested according to the NCI[a] protocols it showed mean $GI_{50}$ of 1.26 $\mu$M. This prompted us to reconsider the possibility of bismethylation, and compounds **13**−**15** were prepared accordingly.
4. Compound **16** was prepared to evaluate the effect of shifting methyl and methoxy group from the chloroindole to the oxindole portion.
5. In the compounds so far described, the best results were obtained when the aldehyde was bearing a methoxy group at the 5 position and a methyl group at the 6 position. Compounds **17**−**22** were prepared to investigate if they could be replaced by different substituents and in particular with a group that can be easily protonated.
6. In the previous paper[6] good results were obtained with an additional condensed benzene ring in the oxindole portion. Compounds **23**−**28** bear an additional condensed benzene ring but in the chloroaldehyde portion.
7. With the synthesis of compounds **29**−**34** we were planning to compare the activity of the 2-chloro with that of the unsubstituted derivatives.
8. The introduction of a hydrophilic and electron attracting group was considered in compounds **35** and **36**.
9. Compound **37** does not belong to the same series considered so far but could be the first term of a new series (devoid of the methine bridge) if it should display significant antitumor activity.

## 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 **4**, **6**, **11**, **24**−**26**, **35**, and **37** a mixture of acetic acid/hydrochloric acid has been employed.

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[a]Abbreviations: NCI, National Cancer Institute; DTP, Developmental Therapeutics Program; GI, growth inhibition; TGI, total growth inhibition; LC, lethal concentration; BEC, Biological Evaluation Committee; MTD, maximum tolerated dose; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; EDTA, ethylenediaminetetraacetic; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PI, propidium iodide.



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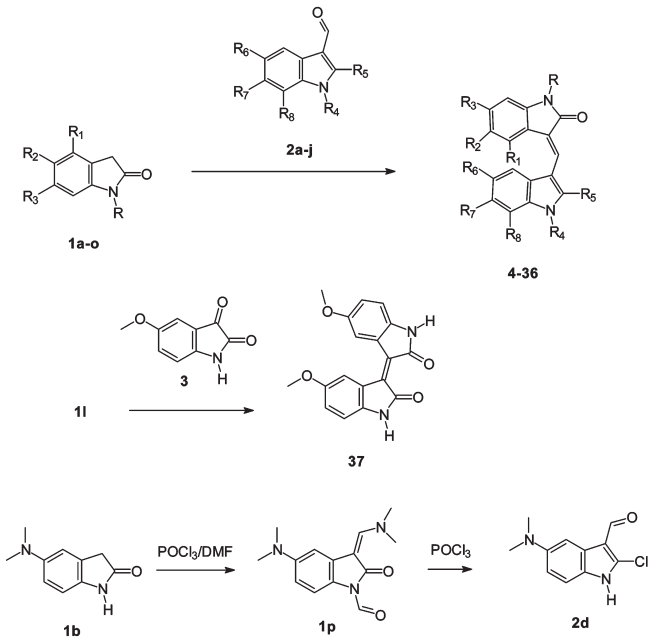
**Scheme 1**




DMA = dimethylamino, Succ = succinyl, CBR = condensed benzene ring.

| Comp | Starting compound **1** | | | | | Starting compound **2** or **3** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **R** | $R_1$ | $R_2$ | $R_3$ | | $R_4$ | $R_5$ | $R_6$ | $R_7$ | $R_8$ |
| **4** | **1a**[9] | H | H | Br | H | **2a**[10] | H | Cl | $OCH_3$ | $CH_3$ | H |
| **5** | **1b**[11] | H | H | DMA | H | **2a** | H | Cl | $OCH_3$ | $CH_3$ | H |
| **6** | **1c**[12] | H | H | Succ | H | **2a** | H | Cl | $OCH_3$ | $CH_3$ | H |
| **7** | **1d** | H | H | H | Cl | **2a** | H | Cl | $OCH_3$ | $CH_3$ | H |
| **8** | **1e**[10] | H | H | $OCH_3$ | $CH_3$ | **2b**[6] | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **9** | **1a** | H | H | Br | H | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **10** | **1b** | H | H | DMA | H | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **11** | **1c** | H | H | Succ | H | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **12** | **1d** | H | H | H | Cl | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **13** | **1f**[13] | $CH_3$ | H | H | H | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **14** | **1g**[14] | $CH_3$ | H | OH | H | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **15** | **1h** | $CH_3$ | H | $OCH_3$ | $CH_3$ | **2b** | $CH_3$ | Cl | $OCH_3$ | $CH_3$ | H |
| **16** | **1h** | $CH_3$ | H | $OCH_3$ | $CH_3$ | **2c**[15] | H | Cl | H | H | H |
| **17** | **1i**[16] | H | Cl | H | H | **2d** | H | Cl | DMA | H | H |
| **18** | **1j** | H | H | Cl | H | **2d** | H | Cl | DMA | H | H |
| **19** | **1k**[17] | H | H | OH | H | **2d** | H | Cl | DMA | H | H |
| **20** | **1l**[18] | H | H | $OCH_3$ | H | **2d** | H | Cl | DMA | H | H |
| **21** | **1g** | $CH_3$ | H | OH | H | **2d** | H | Cl | DMA | H | H |
| **22** | **1b** | H | H | DMA | H | **2d** | H | Cl | DMA | H | H |
| **23** | **1i** | H | Cl | H | H | **2e** | H | Cl | H | CBR | |
| **24** | **1j** | H | H | Cl | H | **2e** | H | Cl | H | CBR | |
| **25** | **1m**[19] | H | H | F | H | **2e** | H | Cl | H | CBR | |
| **26** | **1n**[4] | H | H | OH | $CH_3$ | **2e** | H | Cl | H | CBR | |
| **27** | **1e** | H | H | $OCH_3$ | $CH_3$ | **2e** | H | Cl | H | CBR | |
| **28** | **1g** | $CH_3$ | H | OH | H | **2e** | $CH_3$ | Cl | H | CBR | |
| **29** | **1o** | H | H | H | H | **2f**[20] | H | H | $OCH_3$ | H | H |
| **30** | **1o** | H | H | H | H | **2g**[21] | H | H | $OCH_3$ | $CH_3$ | H |
| **31** | **1e** | H | H | $OCH_3$ | $CH_3$ | **2g** | H | H | $OCH_3$ | $CH_3$ | H |
| **32** | **1e** | H | H | $OCH_3$ | $CH_3$ | **2h** | H | H | H | H | H |
| **33** | **1f** | $CH_3$ | H | H | H | **2g** | H | H | $OCH_3$ | $CH_3$ | H |
| **34** | **1g** | $CH_3$ | H | OH | H | **2g** | H | H | $OCH_3$ | $CH_3$ | H |
| **35**[22] | **1o** | H | H | H | H | **2i** | H | H | COOH | H | H |
| **36** | **1o** | H | H | H | H | **2j** | H | COOH | $OCH_3$ | $CH_3$ | H |
| **37** | **1l** | H | H | $OCH_3$ | H | **3** | | | | | |

**Table 1.** Compounds **4**−**37**

| compd | formula | MW | mp, °C | compd | formula | MW | mp, °C |
|---|---|---|---|---|---|---|---|
| **4** | $C_{19}H_{14}BrClN_2O_2$ | 417.69 | 325−328 dec | **21** | $C_{20}H_{18}ClN_3O_2$ | 367.83 | 200−205 dec |
| **5** | $C_{21}H_{20}ClN_3O_2$ | 381.86 | 295−298 dec | **22** | $C_{21}H_{21}ClN_4O$ | 380.87 | 340−343 dec |
| **6** | $C_{23}H_{19}ClN_2O_5$ | 438.86 | 338−340 | **23** | $C_{21}H_{12}Cl_2N_2O$ | 379.24 | 278−281 dec |
| **7** | $C_{19}H_{14}Cl_2N_2O_2$ | 373.23 | 285−290 dec | **24** | $C_{21}H_{12}Cl_2N_2O$ | 379.24 | 310−312 dec |
| **8** | $C_{22}H_{21}ClN_2O_3$ | 396.87 | 284−286 dec | **25** | $C_{21}H_{12}ClFN_2O$ | 362.79 | 330−332 dec |
| **9** | $C_{20}H_{16}BrClN_2O_2$ | 431.71 | 290−292 dec | **26** | $C_{22}H_{15}ClN_2O_2$ | 374.82 | 185−187 dec |
| **10** | $C_{22}H_{22}ClN_3O_2$ | 395.88 | 230−233 dec | **27** | $C_{23}H_{17}ClN_2O_2$ | 388.85 | 317−320 dec |
| **11** | $C_{24}H_{21}ClN_2O_5$ | 452.89 | 340−345 dec | **28** | $C_{22}H_{15}ClN_2O_2$ | 374.82 | 335−338 dec |
| **12** | $C_{20}H_{16}Cl_2N_2O_2$ | 387.26 | 250−253 dec | **29** | $C_{18}H_{14}N_2O_2$ | 290.32 | 255−258 |
| **13** | $C_{21}H_{19}ClN_2O_2$ | 366.84 | 277−280 dec | **30** | $C_{19}H_{16}N_2O_2$ | 304.34 | 288−290 |
| **14** | $C_{21}H_{19}ClN_2O_3$ | 382.84 | 348−350 dec | **31** | $C_{21}H_{20}N_2O_3$ | 348.40 | 273−275 |
| **15** | $C_{23}H_{23}ClN_2O_3$ | 410.90 | 225−228 | **32** | $C_{19}H_{16}N_2O_2$ | 304.34 | 272−275 |
| **16** | $C_{20}H_{17}ClN_2O_2$ | 352.82 | 220−222 dec | **33** | $C_{20}H_{18}N_2O_2$ | 318.37 | 277−280 |
| **17** | $C_{19}H_{15}Cl_2N_3O$ | 372.25 | 335−340 dec | **34** | $C_{20}H_{18}N_2O_3$ | 334.37 | 280−282 |
| **18** | $C_{19}H_{15}Cl_2N_3O$ | 372.25 | 200−204 dec | **36** | $C_{20}H_{16}N_2O_4$ | 348.35 | 175−180 |
| **19** | $C_{19}H_{16}ClN_3O_2$ | 353.80 | 345−350 dec | **37** | $C_{18}H_{14}N_2O_4$ | 322.32 | 340−345 |
| **20** | $C_{20}H_{18}ClN_3O_2$ | 367.83 | 180−185 dec | | | | |

Most compounds were obtained as almost pure geometrical isomers that, according to the usual NOE experiments described in the previous papers,[7,8] were assigned to the *E* configuration. Compounds **18** and **34** were obtained as evident *E*/*Z* mixtures (around 70/30) and submitted as such to the biological tests. The *E*/*Z* ratio in solution is time dependent and tends to be

50/50. In our experience with similar derivatives, we described the separations of the two isomers by fractional crystallization,[7] but no significant difference in the pharmacological behavior was noticed.

The oxindoles **1d**, **1j**, **1o**, the aldehyde **2h**, and the isatin **3** are commercially available, whereas the other starting compounds (oxindoles and aldehydes) have been prepared according to the literature[4,6,9−22] except **1h**, **2d**, **2e**, and **2j**, reported in the Experimental Section.

## Biology

**a. 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 **5**, **6**, **11**, **17**, **22**, **31**, **35**, **36**, and **37** 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 $\mu$M 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-concentration 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 23.99 and 0.03 $\mu$M, and compounds **7**, **12**, **13**, **14**, **16**, **24**, **27**, **28**, and **30** were submitted to BEC (Biological Evaluation Committee) for possible future development. Further studies continued at the NCI where the maximum tolerated dose (MTD) was determined for compounds **16**, **28**, and **30**. It was found to be 100 mg/kg for compound **16**, whereas it was found to be 400 mg/kg for compounds **28** and **30**, administered ip in DMSO for **16** and **28** and in 10% DMSO in saline/Tween 80 for **30**. Compound **30** was then chosen for the first in vivo experiment, i.e., the "hollow fiber assay",[23] which is a rapid and efficient initial in vivo screening model including multiple cell lines in a single assay. Therefore, it was subjected to four experiments against a panel of three tumor cell lines each consisting of the breast, non-small-cell lung, colon, ovarian, CNS, and melanoma cell lines (administered ip in 10% DMSO and saline/Tween 80). The cancer cell lines were loaded into biocompatible polyvinylidene fluoride hollow fibers. Duplicate sets of fibers were implanted into mice intraperitoneally (ip) and subcutaneously (sc) such that each mouse carried three fibers ip and three fibers sc, representing three distinct cell lines. The mice were treated ip with compound **30** once daily for 4 days, the fibers are collected 24 h after treatment, and the viable cell mass in each fiber is determined.

A point system was developed to assess the activity. A value of 2 is assigned for each treatment dose that results in a 50% or greater reduction in viable cell mass in any fiber. Compounds with a combined ip and sc score of 20 or higher, an sc score of 8 or higher, or cell kill of one or more cell lines are considered for further in vivo testing. Compound **30** resulted in a combined ip + sc score of 16, a sc score of 6, and gave cell kill. Nevertheless, at this time BEC decided to suspend further testing.

In light of the NCI 60 results, the following may be considered: shifting of chlorine from position 4/5 to 6 of the oxindole portion is accompanied by an activity increment which was more evident when the indole portion was N-methylated. In fact compound **12** showed mean $GI_{50}$ of 0.03 $\mu$M vs 5.37 $\mu$M of the 4-chloro isomer and 0.45 $\mu$M of the 5-chloro isomer.[6]

The comparison between the activity of compounds **7** and **12** (mean $GI_{50}$ of 0.21 and 0.03 $\mu$M, respectively) confirms the activity increment observed for some analogues after N-methylation of the chloroaldeyde. To a lesser extent an increment was observed even in the comparison of **4** (mean $GI_{50}$ of 2.14 $\mu$M) vs **9** (1.66 $\mu$M) and **5** (inactive in the preliminary test) vs **10** (11.22 $\mu$M), whereas the same does not happen in the comparison of **8** (23.99 $\mu$M) vs the previously reported NH free analogue (2.00 $\mu$M)[4] and in the comparison of **13**−**15** vs the monomethyl derivatives.[4,5]

Shifting of the methyl and methoxy groups from the chloroindole to the oxindole portion (**16**) did not lead to a significant difference in the biological behavior: mean $GI_{50}$, TGI, $LC_{50}$ of 0.45, 10.96, 56.23 $\mu$M, respectively, vs 0.40, 12.59, 79.43 $\mu$M of the previously reported analogue.[4]

The introduction of the dimethylamino group was accompanied by loss of activity with respect to the 5-methoxy-6-methyl analogues;[5] compound **22**, bearing two dimethylamino groups, was inactive in the preliminary test. Other attempts were unsuccessful such as the introduction of hydrophilic/electron attracting groups or the lack of the methine bridge.

The evaluation of the antitumor activity of the compounds lacking chlorine at the 2-position of the indole portion (**29**−**34**) did not indicate that this class of compounds is superior to the 2-chloro derivatives: only compound **29** was more active than the corresponding 2-chloro analogue (mean $GI_{50}$ of 4.57 vs 77.62 $\mu$M [3]), whereas **30**−**34** were about as active as the chloro analogues. Compound **30** was selected by NCI for in vivo studies.

The replacement of the indole by a benzoindole portion led to the most interesting compounds. In particular, **28** was the most active of the whole series described in this paper, thus confirming that the bulky benzoindole system is a suitable pharmacophoric group in this class of compounds.

**b. Effect on Growth of MCF-7 Breast Cancer Cells.** A subset of compounds was used for further assays in a biological model. First, we examined the influence on the growth and death of MCF-7 breast cancer cells of compounds **12**, **28** (which in the 60-cell panel were very active), and **16**, a less-active compound.

Figure 1A shows that even at a low concentration (200 nM), **12** and **28** blocked MCF-7 cell growth within 48 h. At the same concentration, the less effective compound **16** initially decreased cell proliferation; however, after 24 h, the cells recovered and started to grow. In some preliminary

**Table 2.** Nine Subpanels at Five Concentrations: Growth Inhibition and Cytostatic and Cytotoxic Activity ($\mu$M) of the Selected Compounds (See Supporting Information for the Complete List of Cell Lines Employed)

| compd[a] | modes | leukemia | NSCLC | colon | CNS | melanoma | ovarian | renal | prostate | breast | MG-MID[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **4** | $GI_{50}$ | 1.62 | 3.02 | 1.95 | 1.58 | 2.14 | 2.00 | 2.45 | 2.24 | 2.14 | 2.14 |
| | TGI | 6.17 | 14.13 | 6.76 | 8.91 | 9.55 | 8.13 | 10.96 | 7.94 | 10.47 | 9.33 |
| | $LC_{50}$ | 37.15 | 53.70 | 23.44 | 35.48 | 41.69 | 38.90 | 37.15 | 30.90 | 50.12 | 39.81 |
| **7**[c] | $GI_{50}$ | 0.14 | 0.43 | 0.17 | 0.12 | 0.23 | 0.28 | 0.23 | 0.22 | 0.15 | 0.21 |
| | TGI | 4.79 | 11.48 | 3.55 | 4.37 | 11.48 | 14.79 | 7.08 | 5.25 | 2.63 | 6.61 |
| | $LC_{50}$ | 58.88 | 52.48 | 25.70 | 41.69 | 35.48 | 48.98 | 46.77 | 41.69 | 39.81 | 41.69 |
| **8**[c] | $GI_{50}$ | 18.20 | 34.67 | 22.91 | 26.92 | 15.14 | 30.20 | 41.69 | 22.39 | 18.62 | 23.99 |
| | TGI | 60.26 | 97.72 | 87.10 | 87.10 | 45.71 | 91.20 | | 97.72 | 77.62 | 77.62 |
| | $LC_{50}$ | 97.72 | | | | 57.54 | | | | 95.50 | 91.20 |
| **9**[c] | $GI_{50}$ | 2.51 | 1.91 | 1.55 | 0.93 | 1.55 | 3.47 | 1.70 | 1.55 | 0.95 | 1.66 |
| | TGI | 20.89 | 9.33 | 6.03 | 7.08 | 7.41 | 13.49 | 6.76 | 8.91 | 4.90 | 8.32 |
| | $LC_{50}$ | | 37.15 | 29.51 | 38.90 | 31.62 | 43.65 | 26.92 | 26.30 | 31.62 | 36.31 |
| **10** | $GI_{50}$ | 10.00 | 18.20 | 12.30 | 10.72 | 10.23 | 10.47 | 11.22 | 20.42 | 6.92 | 11.22 |
| | TGI | 44.67 | 91.20 | 70.79 | 60.26 | 67.61 | 77.62 | 69.18 | 97.72 | 54.95 | 67.61 |
| | $LC_{50}$ | 87.10 | | | | 93.33 | 91.20 | | | | 97.72 |
| **12**[c] | $GI_{50}$ | 0.02 | 0.07 | 0.02 | 0.02 | 0.02 | 0.03 | 0.04 | 0.02 | 0.05 | 0.03 |
| | TGI | 1.74 | 11.48 | 0.95 | 1.20 | 7.08 | 2.34 | 15.85 | 3.02 | 5.62 | 3.80 |
| | $LC_{50}$ | 39.81 | 93.33 | 25.12 | 23.44 | 50.12 | 52.48 | 70.79 | 21.38 | 72.44 | 48.98 |
| **13**[c] | $GI_{50}$ | 0.25 | 0.58 | 0.20 | 0.20 | 0.34 | 0.38 | 0.33 | 0.39 | 0.28 | 0.32 |
| | TGI | 4.07 | 33.11 | 8.51 | 4.47 | 14.13 | 18.20 | 10.23 | 10.00 | 13.18 | 11.48 |
| | $LC_{50}$ | 46.77 | | 72.44 | 74.13 | 69.18 | 95.50 | 79.43 | 66.07 | 95.50 | 77.62 |
| **14**[c] | $GI_{50}$ | 0.05 | 0.22 | 0.07 | 0.04 | 0.11 | 0.26 | 0.11 | 0.10 | 0.06 | 0.10 |
| | TGI | 0.47 | 6.03 | 3.55 | 1.74 | 7.41 | 11.75 | 4.27 | 3.16 | 1.70 | 3.39 |
| | $LC_{50}$ | 19.95 | 42.66 | 26.92 | 29.51 | 33.11 | 47.86 | 36.31 | 23.99 | 40.74 | 33.88 |
| **15** | $GI_{50}$ | 6.92 | 19.50 | 20.42 | 10.47 | 16.22 | 18.62 | 15.85 | 35.48 | 6.76 | 14.13 |
| | TGI | 56.23 | 69.18 | 83.18 | 26.92 | 67.61 | 60.26 | 41.69 | 67.61 | 36.31 | 52.48 |
| | $LC_{50}$ | | 93.33 | 97.72 | 61.66 | | 89.13 | 77.62 | | 83.18 | 87.10 |
| **16**[c] | $GI_{50}$ | 0.26 | 0.58 | 0.37 | 0.32 | 0.71 | 0.60 | 0.62 | 0.32 | 0.30 | 0.45 |
| | TGI | 7.41 | 12.30 | 9.33 | 7.59 | 18.20 | 11.75 | 12.88 | 21.38 | 8.32 | 10.96 |
| | $LC_{50}$ | 69.18 | 57.54 | 46.77 | 47.86 | 51.29 | 61.66 | 56.23 | 69.18 | 57.54 | 56.23 |
| **18**[c] | $GI_{50}$ | 1.62 | 2.57 | 2.09 | 1.58 | 2.69 | 3.31 | 1.78 | 2.45 | 1.32 | 2.09 |
| | TGI | 17.78 | 10.00 | 7.24 | 5.50 | 11.22 | 11.75 | 8.13 | 13.49 | 9.12 | 9.77 |
| | $LC_{50}$ | 91.20 | 46.77 | 22.91 | 18.20 | 37.15 | 41.69 | 29.51 | 46.77 | 54.95 | 38.90 |
| **19** | $GI_{50}$ | 2.24 | 5.13 | 3.89 | 3.80 | 5.25 | 7.24 | 5.13 | 8.91 | 2.88 | 4.37 |
| | TGI | 13.49 | 31.62 | 20.42 | 36.31 | 25.70 | 52.48 | 33.88 | 53.70 | 33.88 | 29.51 |
| | $LC_{50}$ | | 95.50 | 69.18 | | 79.43 | | 91.20 | | | 91.20 |
| **20**[c] | $GI_{50}$ | 1.62 | 3.02 | 1.55 | 2.09 | 3.55 | 3.39 | 3.02 | 2.88 | 1.15 | 2.29 |
| | TGI | 10.23 | 15.49 | 9.55 | 7.94 | 15.49 | 15.14 | 13.80 | 19.05 | 8.51 | 12.02 |
| | $LC_{50}$ | 72.44 | 58.88 | 30.20 | 32.36 | 44.67 | 50.12 | 52.48 | 50.12 | 54.95 | 48.98 |
| **21** | $GI_{50}$ | 16.60 | 6.61 | 4.90 | 6.03 | 4.57 | 8.32 | 4.37 | 12.88 | 3.80 | 6.03 |
| | TGI | | 46.77 | 20.89 | 33.88 | 26.30 | 54.95 | 31.62 | | 30.90 | 38.02 |
| | $LC_{50}$ | | 85.11 | 61.66 | 85.11 | 66.07 | 95.50 | 87.10 | | | 83.18 |
| **23**[c] | $GI_{50}$ | 5.50 | 9.55 | 4.90 | 3.63 | 4.57 | 2.51 | 8.91 | 10.23 | 5.01 | 5.50 |
| | TGI | 25.70 | 28.18 | 17.38 | 17.78 | 19.05 | 13.18 | 23.44 | 31.62 | 19.95 | 20.89 |
| | $LC_{50}$ | 75.86 | 72.44 | 47.86 | 53.70 | 46.77 | 57.54 | 56.23 | 63.10 | 67.61 | 58.88 |
| **24**[c] | $GI_{50}$ | 0.19 | 0.87 | 0.49 | 0.36 | 0.66 | 0.59 | 0.55 | 0.38 | 0.38 | 0.49 |
| | TGI | 7.24 | 12.59 | 6.17 | 5.37 | 11.22 | 11.22 | 8.51 | 8.91 | 5.62 | 8.32 |
| | $LC_{50}$ | 72.44 | 58.88 | 35.48 | 47.86 | 40.74 | 61.66 | 52.48 | 38.02 | 54.95 | 51.29 |
| **25**[c] | $GI_{50}$ | 0.49 | 0.65 | 0.35 | 0.40 | 0.56 | 0.33 | 0.63 | 0.39 | 0.40 | 0.47 |
| | TGI | 8.71 | 12.59 | 6.92 | 6.46 | 10.96 | 7.41 | 10.96 | 9.77 | 6.76 | 8.91 |
| | $LC_{50}$ | 87.10 | 70.79 | 57.54 | 51.29 | 44.67 | 41.69 | 43.65 | 51.29 | 70.79 | 56.23 |
| **26** | $GI_{50}$ | 3.31 | 7.94 | 6.61 | 8.71 | 6.46 | 9.12 | 8.71 | 5.37 | 3.72 | 6.31 |
| | TGI | 38.02 | 30.20 | 20.89 | 24.55 | 20.89 | 46.77 | 33.11 | 26.30 | 19.05 | 26.92 |
| | $LC_{50}$ | | 81.28 | 61.66 | 67.61 | 57.54 | 87.10 | 75.86 | 74.13 | 69.18 | 72.44 |
| **27**[c] | $GI_{50}$ | 0.19 | 0.63 | 0.31 | 0.31 | 0.56 | 0.40 | 0.49 | 0.25 | 0.23 | 0.37 |
| | TGI | 1.00 | 9.33 | 2.88 | 3.39 | 7.94 | 7.24 | 8.13 | 3.02 | 4.37 | 4.79 |
| | $LC_{50}$ | 19.05 | 47.86 | 18.62 | 30.90 | 32.36 | 33.88 | 35.48 | 25.70 | 47.86 | 30.90 |
| **28**[c] | $GI_{50}$ | 0.01 | 0.06 | 0.02 | 0.02 | 0.03 | 0.04 | 0.04 | 0.01 | 0.02 | 0.03 |
| | TGI | 0.08 | 4.07 | 0.71 | 1.95 | 4.68 | 4.68 | 5.62 | 0.68 | 1.32 | 1.78 |
| | $LC_{50}$ | 5.75 | 32.36 | 9.12 | 22.39 | 27.54 | 27.54 | 51.29 | 16.22 | 66.07 | 23.99 |
| **29** | $GI_{50}$ | 2.34 | 5.13 | 2.24 | 6.17 | 6.61 | 6.31 | 5.75 | 5.62 | 3.47 | 4.57 |
| | TGI | 7.76 | 25.12 | 15.49 | 28.84 | 27.54 | 30.20 | 26.30 | 23.99 | 18.20 | 21.38 |
| | $LC_{50}$ | 43.65 | 77.62 | 70.79 | 75.86 | 75.86 | 81.28 | 83.18 | 83.18 | 91.20 | 74.13 |
| **30**[c] | $GI_{50}$ | 0.09 | 0.11 | 0.09 | 0.15 | 0.12 | 0.18 | 0.23 | 0.07 | 0.12 | 0.13 |
| | TGI | 2.88 | 16.98 | 3.98 | 3.63 | 7.41 | 7.76 | 6.92 | 12.88 | 6.76 | 6.46 |
| | $LC_{50}$ | 64.57 | 77.62 | 22.91 | 53.70 | 50.12 | 58.88 | 53.70 | 63.10 | 75.86 | 54.95 |

**Table 2.** Continued

| compd[a] | modes | leukemia | NSCLC | colon | CNS | melanoma | ovarian | renal | prostate | breast | MG-MID[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **32** | $GI_{50}$ | 8.71 | 13.49 | 8.13 | 10.00 | 13.18 | 12.59 | 11.48 | 13.80 | 11.75 | 11.22 |
| | TGI | 54.95 | 43.65 | 52.48 | 33.88 | 37.15 | 45.71 | 38.02 | 36.31 | 40.74 | 42.66 |
| | $LC_{50}$ | | 89.13 | 95.50 | 95.50 | 81.28 | 97.72 | 85.11 | 91.20 | 91.20 | 91.20 |
| **33**[c] | $GI_{50}$ | 2.00 | 6.31 | 0.76 | 1.07 | 3.09 | 7.76 | 4.17 | 4.17 | 1.78 | 2.75 |
| | TGI | 35.48 | 66.07 | 60.26 | 42.66 | 74.13 | 60.26 | 60.26 | 81.28 | 54.95 | 57.54 |
| | $LC_{50}$ | 93.33 | 93.33 | 97.72 | 95.50 | 97.72 | | | | | 97.72 |
| **34** | $GI_{50}$ | 3.47 | 6.03 | 2.95 | 3.72 | 3.24 | 5.62 | 5.89 | 3.55 | 2.75 | 4.07 |
| | TGI | 36.31 | 21.88 | 24.55 | 23.99 | 15.14 | 31.62 | 28.18 | 15.85 | 16.22 | 22.91 |
| | $LC_{50}$ | | 91.20 | | 67.61 | 66.07 | 95.50 | 93.33 | | 63.10 | 83.18 |
| 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 concentration = $10^{-4}$ M unless otherwise reported. Only values <100 $\mu$M 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 concentration = $10^{-3}$ M.

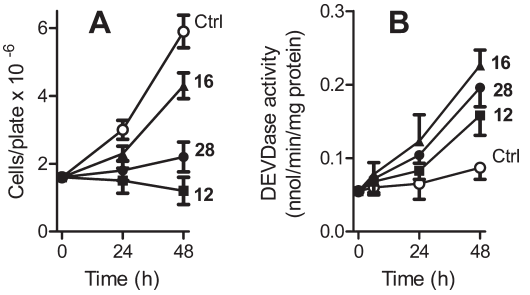



**Figure 1.** Effects on growth and death of MCF-7 cells. (A) Rate of cell growth determined as total cell number. The cells were incubated in the presence of the indicated compounds (200 nM), and viable cells were counted daily. The differences of all treated cells vs control cells were significant ($P < 0.05$) at either 24 and 48 h. (B) Caspase activity acting on the peptide sequence DEVD (mainly caspase 7) was measured in extracts obtained from cells treated for 24 or 48 h with the indicated compounds (5 $\mu$M). The reported data are mean values ± SEM obtained in five determinations. The differences of all treated cells vs control cells were significant ($P < 0.05$) only at 48 h.

experiments, apoptotic cell death was observed after a longer treatment time (72−96 h). At shorter times, a significant toxic effect became evident only at concentrations in the micromolar range, and cell death was detectable after a 48 h incubation (data not shown). To understand whether the toxicity of these new derivatives was associated with activation of apoptosis, the cells were treated with a 5 $\mu$M concentration of the compounds, a concentration that caused about 30−40% of cell death, and the activation of caspases acting on the peptide substrate sequence Asp-Glu-Val-Asp (DEVD) was determined. This sequence is the optimal substrate for effector caspases 3 and 7, activated as the result of all caspase cascades.[24] However, since MCF-7 cells lack caspase 3, the assay measures mainly the activation of caspase 7, which is highly expressed in these cells.[25] The activation of the caspase proteases, considered the molecular effectors of apoptosis,[26] was significant only after 48 h of treatment (Figure 1B).

We next examined the effect of the derivatives on some biochemical pathways correlated with cell proliferation and cell death (Figure 2). In these studies, the cells were treated with the different agents at 5 $\mu$M for 20 h. First, we determined whether the antiproliferative effect was accompanied by changes in the expression of the tumor suppressor p53. Figure 2A shows that compounds **12**, **16**, and **28** caused a p53 increase. Next, we investigated the effect on ERK1/2

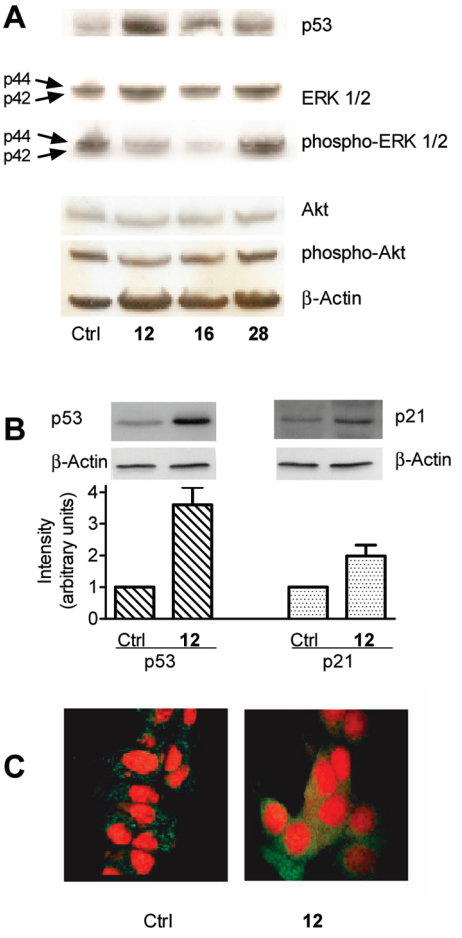



**Figure 2.** Effect on biochemical pathways correlated with cell growth in MCF-7 cells. (A) The cells were incubated for 20 h in the presence of 5 $\mu$M of the indicated compound. Then the content and phosphorylation status of the indicated proteins were determined in cell extracts by Western blotting (80 $\mu$g of protein/lane). Immunoblots reported are from one experiment representative of at least three that gave similar results. (B) Quantitative determination of p53 and p21 proteins by densitometry of immunoblotting in cells incubated 24 h in the presence of 5 $\mu$M compound **12**. Results are mean values ± SEM obtained in four separate determinations. Differences between treated cells and control cells were significant ($P < 0.05$) for both p53 and p21. (C) Effect of compound **12** (5 $\mu$M for 24 h) on cellular Bax detected by immunofluorescence confocal microscopy. Nuclei were evidenced by incubation with propidium iodide (red fluorescence). Bax was stained with FITC antibody and is evidenced as green fluorescence. The image is representative of three experiments.

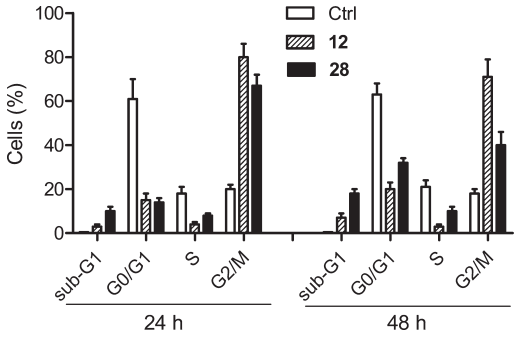



**Figure 3.** Effects on cell cycle in MCF-7 cells. Cells were treated with 200 nM of the indicated compound for 24 h. Afterward cell cycle distribution was determined by flow cytometry. Results are mean values ± SEM of three determinations.

mitogen-activated protein kinases, which are generally associated with tumor cell growth.[27] In this case, the three compounds had different effects. In fact, **12** and especially **16** decreased ERK phosphorylation which, on the contrary, was increased by compound **28**. The three derivatives had minimal effects on the kinase Akt, an oncoprotein upregulated in several cancers and responsible for resistance to cell death,[28] Akt-phosphorylation was only slightly decreased by compound **12**.

These data indicate that interference in the function of growth-related kinases ERKs and Akt does not represent a common mechanism of the antiproliferative effect of the tested compounds, whereas p53 appears correlated to the action of these derivatives. To confirm the involvement of p53, the effects of the potent compound **12** on the levels of the p53 transcriptional target p21[29] were determined. In cells incubated 24 h with **12**, together with a 3-fold induction of p53, p21 was also increased by about 90% (Figure 2B). Another important protein associated with the tumor suppressive role of p53 is Bax, which is an effector of p53-induced apoptosis and is a transcriptional target of p53 and a partner of p53 in its nontranscriptional actions.[30] The cellular content of the Bax protein was assayed by immunofluorescence microscopy. Figure 2C shows that treatment with compound **12** caused a considerable Bax accumulation, evidenced by a large increase in green fluorescence. Densitometric analysis of bands obtained by Western blotting ($n = 3$) indicated an increase of 2.8 times (data not shown).

The p53 tumor suppressor can cause apoptosis and influence the cell cycle.[31] The increase of p21 caused by **12** suggested that the anticancer action of the novel compounds could also be associated with interference in cell cycle progression. Therefore, we examined the effect of some derivatives on the cell cycle profile of MCF-7 cells. The cells were incubated for 24 and 48 h in the presence of 200 nM **12** and **28**, and the analysis of DNA profiles was performed by flow cytometry. As previously detailed, at this concentration both compounds strongly inhibit cell growth without causing massive cell death. Figure 3 shows that they caused a marked accumulation of MCF-7 cells in the G2/M phase, whereas most of the control cells were in the G0/G1. Moreover, after treatment with the analogues, a notable cell population was observed in a sub-G1 peak caused by fragmented DNA in apoptotic cells. This population increased with incubation time, confirming activation of apoptosis.

In conclusion, the most active compounds tested on MCF-7 breast cancer cells gave rise to a block in cell cycle progression, with cell arrest in the G2/M phase. The arrest of the cell cycle probably causes the late induction of apoptosis, as indicated by activation of caspase proteases and appearance of a sub-G1 cell population. From the biochemical point of view, these events can be explained by the induction of the tumor suppressor p53, which is known to exert cell cycle blocking effect and eventual activation of apoptosis directly and by means of several effectors such as p21 or Bax,[31] which were also increased.

## Experimental Section

**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.; $\nu_{max}$ is expressed in $cm^{-1}$. The $^1H$ NMR spectra were recorded on a Varian Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in $\delta$ (ppm) and $J$ in Hz (abbreviations: ar = aromatic, bzind = benzoindole, ind = indole, ox = oxindole). For the spectra that are not reported here, see Supporting Information. The oxindoles **1d**, **1j**, **1o**, the aldehyde **2h** and the isatin **3** are commercially available. The following compounds were prepared according to the literature: **1a**,[9] **1b**,[11] **1c**,[12] **1e**,[10] **1f**,[13] **1g**[14] **1i**,[16] **1k**,[17] **1l**,[18] **1m**,[19] **1n**,[4] **2a**,[10] **2b**,[6] **2c**,[15] **2f**,[20] **2g**,[21] and **35**.[22]

**Synthesis of 5-Methoxy-1,6-dimethyl-1,3-dihydro-2*H*-indol-2-one (1h).** 5-Methoxy-6-methyl-1,3-dihydro-2*H*-indol-2-one[10] (4 mmol) in acetone (40 mL) was treated with dimethyl sulfate (9.5 mmol) in the presence of anhydrous potassium carbonate (6.5 mmol). The reaction mixture was refluxed for 15 h, cooled, and filtered. The solution was evaporated and the expected compound was purified by column chromatography (petroleum ether/acetone, 70/30), with a yield of 52%. It was crystallized from acetone/petroleum ether: mp 93−95 °C. IR: 1697, 1240, 1151, 1069. $^1H$ NMR: 2.17 (3H, s, $CH_3$), 3.06 (3H, s, N-$CH_3$), 3.47 (2H, s, $CH_2$), 3.73 (3H, s, $OCH_3$), 6.79 (1H, s, ar), 6.94 (1H, s, ar). Anal. Calcd for $C_{11}H_{13}NO_2$ (MW 191.23): C, 69.09; H, 6.85; N, 7.32. Found: C, 69.12; H, 6.78; N, 7.36.

**Synthesis of 5-(Dimethylamino)-3-[(dimethylamino)methylene]-2-oxoindoline-1-carbaldehyde (1p).** The Vilsmeier reagent was prepared at 0−5 °C by dropping $POCl_3$ (32 mmol) into a stirred solution of DMF (43 mmol) in $CHCl_3$ (5 mL). 5-(Dimethylamino)-1,3-dihydroindol-2-one[11] (**1b**) (6 mmol) was dissolved in $CHCl_3$ and treated with the Vislmeier reagent. After 3 h at room temperature, $CHCl_3$ was evaporated under reduced pressure and the residue was poured into ice−water. After neutralization with $NaHCO_3$ the expected compound was collected by filtration and crystallized from EtOH with a yield of 82%: mp 154−157 °C. IR: 1671, 1703, 1177, 1108. $^1H$ NMR: 2.89 (6H, s, $2CH_3$), 3.37 (3H, s, $CH_3$), 3.56 (3H, s, $CH_3$), 6.40 (1H, dd, ind-6, $J = 8.6$, $J = 2.6$), 6.85 (1H, d, ind-4, $J = 2.6$), 7.69 (1H, d, ind-7, $J = 8.6$), 7.85 (1H, s, CH), 9.14 (1H, s, CH). Anal. Calcd for $C_{14}H_{17}N_3O_2$ (MW 259.31): C, 64.85; H, 6.61; N, 16.20. Found: C, 64.87; H, 6.58; N, 16.23.

**Synthesis of 2-Chloro-5-(dimethylamino)indole-3-carbaldehyde (2d).** Compound **1p** (2 mmol) was refluxed for 15 min with $POCl_3$ (22 mmol). The mixture was cooled, poured into ice−water, and basified with 30% $NH_4OH$.[32] The aldehyde **2d** was collected by filtration with a yield of 30%: mp 180−190 °C dec. IR: 3125, 1634, 830, 787. $^1H$ NMR: 2.92 (6H, s, $2CH_3$), 6.87 (1H, dd, ind-6, $J = 8.7$, $J = 2.4$), 7.28 (1H, d, ind-7, $J = 8.7$), 7.39 (1H, d, ind-4, $J = 2.4$), 9.95 (1H, s, CHO), 12.72 (1H, broad, NH). Anal. Calcd for $C_{11}H_{11}ClN_2O$ (MW 222.67): C, 59.33; H, 4.98; N, 12.58. Found: C, 59.29; H, 5.01; N, 12.60.

**Synthesis of 2-Chloro-1*H*-benzo[*g*]indole-3-carbaldehyde (2e) and 3-Formyl-5-methoxy-6-methyl-1*H*-indole-2-carboxylic Acid (2j).** The Vilsmeier reagent was prepared at 0−5 °C by dropping $POCl_3$ (54 mmol) into a stirred solution of DMF (65 mmol) in

$CHCl_3$ (5 mL). The 1,3-dihydro-2*H*-benzo[*g*]indol-2-one, prepared as reported in the literature,[33] or 5-methoxy-6-methylindole-2-carboxylic acid[34] (5 mmol) was suspended in $CHCl_3$ (20 mL) and treated with the Vislmeier reagent. The reaction mixture was kept under reflux for 10−15 h, according to a TLC test. Chloroform was removed under reduced pressure, and the resulting oil was poured into ice. The crude aldehyde thus obtained was collected by filtration with a yield of 70%.

**Data for 2e.** Crystallized from ethanol: mp 220−225 °C. IR: 3160, 1649, 856, 805. $^1$H NMR: 7.52 (1H, t, ar, $J$ = 7.9), 7.64 (1H, t, ar, $J$ = 7.9), 7.73 (1H, d, ar, $J$ = 7.9), 8.00 (1H, d, ar, $J$ = 7.9), 8.16 (1H, d, ar, $J$ = 7.9), 8.38 (1H, d, ar, $J$ = 7.9), 10.08 (1H, s, CHO), 13.77 (1H, s, NH). Anal. Calcd for $C_{13}H_8ClNO$ (MW 229.66): C, 67.99; H, 3.51; N, 6.10. Found: C, 68.02; H, 3.49; N, 6.13.

**Data for 2j.** Crystallized from methanol: mp 260 °C dec. IR: 3247, 1672, 1643, 1102, 1025. $^1$H NMR: 2.26 (3H, s, $CH_3$), 3.83 (3H, s, $OCH_3$), 7.30 (1H, s, ind-4/7), 7.64 (1H, s, ind-4/7), 10.60 (1H, s, CHO), 12.52 (1H, s, NH), 13.20 (1H, broad, COOH). Anal. Calcd for $C_{12}H_{11}NO_4$ (MW 233.22): C, 61.80; H, 4.75; N, 6.01. Found: C, 61.82; H, 4.73; N, 5.98.

**General Procedure for the Synthesis of Compounds 5, 7−10, 12−23, 27−34, and 36.** The appropriate 2-indolinone **1** (10 mmol) was dissolved in methanol (100 mL) and treated with the equivalent of the appropriate indole-3-carbaldehyde **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 with a yield of 20% for compounds **5**, **7**, **28**, and **33**, 45−50% for compounds **13**, **15**, **16**, **23**, **27**, and **31**, and 75−85% for compounds **8**−**10**, **12**, **14**, **29**, **30**, **32**, **34**, and **36**.

Compounds **17**−**22** were purified by column chromatography with a yield of 20%.

**Data for 7.** IR: 3355, 1667, 1593, 1207, 917. $^1$H NMR: 2.25 (3H, s, $CH_3$), 3.56 (3H, s, $OCH_3$), 6.47 (1H, s, ind-4), 6.76 (1H, d, ox-4, $J$ = 8.2), 6.89 (1H, d, ox-7, $J$ = 1.8), 6.95 (1H, dd, ox-5, $J$ = 8.2, $J$ = 1.8), 7.23 (1H, s, ind-7), 7.69 (1H, s, CH), 10.71 (1H, s, NH-ox), 12.60 (1H, broad, NH-ind). Anal. ($C_{19}H_{14}Cl_2N_2O_2$) C, H, N.

**Data for 9.** IR: 3150, 1692, 1594, 1298, 922. $^1$H NMR: 2.32 (3H, s, $CH_3$), 3.62 (3H, s, N-$CH_3$), 3.86 (3H, s, $OCH_3$), 6.48 (1H, s, ind-4), 6.86 (2H, m, ind + ox), 7.35 (1H, d, ox, $J$ = 8.4), 7.52 (1H, s, ox), 7.74 (1H, s, CH), 10.73 (1H, s, NH-ox). Anal. ($C_{20}H_{16}BrClN_2O_2$) C, H, N.

**Data for 12.** IR: 3140, 1714, 1611, 1040, 799. $^1$H NMR: 2.29 (3H, s, $CH_3$), 3.56 (3H, s, N-$CH_3$), 3.82 (3H, s, $OCH_3$), 6.50 (1H, s, ind-4), 6.74 (1H, d, ox-4, $J$ = 7.5), 6.89 (1H, d, ox-7, $J$ = 1.2), 6.92 (1H, dd, ox-5, $J$ = 7.5, $J$ = 1.2), 7.48 (1H, s, ind-7), 7.68 (1H, s, CH), 10.74 (1H, s, NH-ox). Anal. ($C_{20}H_{16}Cl_2N_2O_2$) C, H, N.

**Data for 13.** IR: 1709, 1605, 1040, 912, 851. $^1$H NMR: 2.30 (3H, s, $CH_3$), 3.34 (3H, s, N-$CH_3$), 3.53 (3H, s, N-$CH_3$), 3.84 (3H, s, $OCH_3$), 6.52 (1H, s, ind-4), 6.83 (1H, d, ox-4/7, $J$ = 7.2), 6.94 (1H, t, ox-5/6, $J$ = 7.2), 7.05 (1H, d, ox-4/7, $J$ = 7.2), 7.29 (1H, t, ox-5/6, $J$ = 7.2), 7.49 (1H, s, ind-7), 7.73 (1H, s, CH). Anal. ($C_{21}H_{19}ClN_2O_2$) C, H, N.

**Data for 14.** IR: 3155, 1667, 1592, 1119, 912. $^1$H NMR: 2.31 (3H, s, $CH_3$), 3.19 (3H, s, N-$CH_3$), 3.60 (3H, s, N-$CH_3$), 3.84 (3H, s, $OCH_3$), 6.40 (1H, d, ox-4, $J$ = 2.3), 6.57 (1H, s, ind-4), 6.68 (1H, dd, ox-6, $J$ = 8.2, $J$ = 2.3), 6.83 (1H, d, ox-7, $J$ = 8.2), 7.48 (1H, s, ind-7), 7.68 (1H, s, CH), 8.94 (1H, s, OH). Anal. ($C_{21}H_{19}ClN_2O_3$) C, H, N.

**Data for 16.** IR: 1686, 1602, 1052, 897, 739. $^1$H NMR: 2.17 (3H, s, $CH_3$), 3.20 (3H, s, N-$CH_3$), 3.25 (3H, s, $OCH_3$), 6.43 (1H, s, ox), 6.88 (1H, s, ox), 7.22 (3H, m, ind), 7.46 (1H, d, ind, $J$ = 7.8), 7.64 (1H, s, CH), 12.78 (1H, broad, NH-ind). Anal. ($C_{20}H_{17}ClN_2O_2$) C, H, N.

**Data for 18.** IR: 3165, 1692, 1600, 1215, 804. $^1$H NMR: 2.74 (6H, s, $N(CH_3)_2$), 6.20 (1H, d, ox/ind-4, $J$ = 2.2), 6.70 (1H, d, ox/ind-4, $J$ = 2.2), 6.89 (1H, d, ox/ind-7, $J$ = 8.4), 6.92 (1H, dd, ox/ind-6, $J$ = 8.4, $J$ = 2.2), 7.21 (1H, dd, ox/ind-6, $J$ = 8.4, $J$ = 2.2), 7.34 (1H, d, ox/ind-7, $J$ = 8.4), 7.74 (1H, s, CH), 10.69 (1H, s, NH-ox), 12.60 (1H, broad, NH-ind). Anal. ($C_{19}H_{15}Cl_2N_3O$) C, H, N.

**Data for 20.** IR: 3135, 1683, 1608, 1199, 810. $^1$H NMR: 2.72 (6H, s, $N(CH_3)_2$), 3.43 (3H, s, $OCH_3$), 6.25 (1H, d, ind/ox-4, $J$ = 2.2), 6.39 (1H, s, ind/ox), 6.77 (2H, s, ind/ox), 6.89 (1H, dd, ind/ox-6, $J$ = 9, $J$ = 2.2), 7.31 (1H, d, ind/ox-7, $J$ = 9), 7.65 (1H, s, CH), 10.36 (1H, s, NH-ox), 12.70 (1H, broad, NH-ind). Anal. ($C_{20}H_{18}ClN_3O_2$) C, H, N.

**Data for 27.** IR: 3408, 3172, 1721, 1674, 1086. $^1$H NMR: 2.11 (3H, s, $CH_3$), 3.17 (3H, s, $OCH_3$), 6.49 (1H, s, ox), 6.68 (1H, s, ox), 7.31 (1H, d, bzind, $J$ = 8.1), 7.48 (2H, t, bzind, $J$ = 8.1), 7.61 (2H, m, bzind + CH), 7.96 (1H, d, bzind, $J$ = 8.1), 8.42 (1H, d, bzind, $J$ = 8.1), 10.35 (1H, s, NH-ox), 13.46 (1H, broad, NH-bzind). Anal. ($C_{23}H_{17}ClN_2O_2$) C, H, N.

**Data for 28.** IR: 3200, 1666, 1583, 805, 671. $^1$H NMR: 3.19 (3H, s, $CH_3$), 6.39 (1H, d, ox-4, $J$ = 2.3), 6.68 (1H, dd, ox-6, $J$ = 8.4, $J$ = 2.3), 6.84 (1H, d, ox-7, $J$ = 8.4), 7.28 (1H, d, bzind, $J$ = 8.1), 7.50 (1H, t, bzind, $J$ = 8.1), 7.60 (1H, d, bzind, $J$ = 8.1), 7.62 (1H, t, bzind, $J$ = 8.1), 7.70 (1H, s, CH), 7.99 (1H, d, bzind, $J$ = 8.1), 8.39 (1H, d, bzind, $J$ = 8.1), 8.82 (1H, s, OH), 13.45 (1H, s, NH-bzind). Anal. ($C_{22}H_{15}ClN_2O_2$) C, H, N.

**Data for 30.** IR: 3364, 3131, 1684, 1603, 1100. $^1$H NMR: 2.27 (3H, s, $CH_3$), 3.93 (3H, s, $OCH_3$), 6.83 (1H, d, ox-4/7, $J$ = 7.5), 6.98 (1H, t, ox-5/6, $J$ = 7.5), 7.12 (1H, t, ox-5/6, $J$ = 7.5), 7.27 (1H, s, ind), 7.67 (1H, s, ind), 7.92 (1H, d, ox-4/7), 8.14 (1H, s, CH), 9.33 (1H, s, ind-2), 10.47 (1H, s, NH-ox), 11.78 (1H, broad, NH-ind). Anal. ($C_{19}H_{16}N_2O_2$) C, H, N.

**General Procedure for the Synthesis of Compounds 4, 6, 11, 24−26, and 37.** The appropriate oxindole **1** (5 mmol) was dissolved in acetic acid (25 mL) and treated with an equivalent of indole-3-carbaldehyde **2** or 5-methoxyindole-2,3-dione **3** (for compound **37**) and 37% hydrochloric acid (1 mL). The reaction mixture was refluxed for 24 h, and after it was cooled, the precipitate was collected by filtration with a yield of 35−40% for compounds **4** and **24**−**26** and 70−80% for compounds **6**, **11**, and **37**.

**Data for 4.** IR: 3124, 1672, 1604, 1108, 1021. $^1$H NMR: 2.28 (3H, s, $CH_3$), 3.62 (3H, s, $OCH_3$), 6.48 (1H, s, ind-4), 6.86 (1H, d, ox-7, $J$ = 8.2), 6.90 (1H, d, ox-4, $J$ = 1.5), 7.27 (1H, s, ind-7), 7.36 (1H, dd, ox-6, $J$ = 8.2, $J$ = 1.5), 7.73 (1H, s, CH), 10.74 (1H, s, NH-ox), 12.77 (1H, broad, NH-ind). Anal. ($C_{19}H_{14}BrClN_2O_2$) C, H, N.

**Data for 24.** IR: 3426, 1698, 1623, 1198, 809. $^1$H NMR: 6.79 (1H, d, ox-4, $J$ = 2.3), 6.89 (1H, d, ox-7, $J$ = 8.3), 7.22 (1H, dd, ox-6, $J$ = 8.3, $J$ = 2.3), 7.28 (1H, d, bzind, $J$ = 8.2), 7.51 (2H, t, bzind, $J$ = 8.2), 7.63 (1H, d, bzind, $J$ = 8.2), 7.77 (1H, s, CH), 8.00 (1H, d, bzind, $J$ = 8.2), 8.43 (1H, d, bzind, $J$ = 8.2), 10.76 (1H, s, NH-ox), 13.55 (1H, broad, NH-bzind). Anal. ($C_{21}H_{12}Cl_2N_2O$) C, H, N.

**Data for 25.** IR: 3200, 1607, 1170, 815, 682. $^1$H NMR: 6.57 (1H, dd, ox, $J$ = 9, $J$ = 2.7), 6.88 (1H, dd, ox, $J$ = 8, $J$ = 4.5), 7.04 (1H, td, ox, $J$ = 9, $J$ = 2.7), 7.30 (1H, d, bzind, $J$ = 8.3), 7.53 (2H, m, bzind), 7.63 (1H, d, bzind, $J$ = 8.3), 7.77 (1H, s, CH), 8.00 (1H, d, bzind, $J$ = 8.3), 8.44 (1H, d, bzind, $J$ = 8.3), 10.65 (1H, s, NH-ox), 13.56 (1H, broad, NH-bzind). Anal. ($C_{21}H_{12}ClFN_2O$) C, H, N.

**Biology. a. Cell-Based Screening Assay.** The NCI screening is a two-stage process,[35] beginning with the evaluation of all compounds against the 60 cell lines at a single concentration of $10^{-5}$ M. Compounds exhibiting significant growth inhibition were evaluated against the 60-cell panel at five concentration levels by the NCI according to standard procedures (http://dtp.nci.nih.gov/branches/btb/ivclsp.html). In both cases the exposure time was 48 h.

**b. Acute Toxicity.** To a single female athymic nude mouse (APA) was given a single injection of 400 mg/kg compounds **16**, **28**, and **30**. A second mouse received a dose of 200 mg/kg compounds **16**, **28**, and **30**, and a third mouse received a dose of 100 mg/kg compounds **16**, **28**, and **30**. The mice were allowed ad libitum feed and water, and they were observed for 2 weeks.

The doses of compound **16** were administered ip in DMSO. After 14 days the mice treated with 400 and 200 mg/kg were sacrificed, since they had lost more than 20% of their body weight. Therefore, the MTD was 100 mg/kg. The doses of compound **28** and **30** were administered ip in DMSO and DMSO in saline/Tween 80, respectively. After 14 days no mice showed weight loss or other signs of significant toxicity; therefore, MTD was 400 mg/kg.

**c. Hollow Fiber Assay.** Twelve tumor cell lines (NCI-H23, NCI-H522, MDA-MB-231, MDA-MB-435, SW-620, COLO 205, LOX, UACC-62, OVCAR-3, OVCAR-5, U251, and SF-295) were cultivated in RPMI-1640 containing 10% FBS and 2 mM glutamine. On the day preceding hollow fiber preparation, the cells were given a supplementation of fresh medium to maintain log phase growth. For fiber preparation, the cells were harvested by standard trypsinization technique and resuspended at the desired cell density. The cell suspension was flushed into 1 mm (internal diameter) polyvinylidene fluoride hollow fibers with a molecular weight exclusion of 500 000 Da. The hollow fibers were heat-sealed at 2 cm intervals, and the samples generated from these seals were placed into tissue culture medium and incubated at 37 °C in 5% $CO_2$ for 24−48 h prior to implantation. A total of three different tumor lines were prepared for each experiment so that each mouse received three intraperitoneal implants (one of each tumor line) and three subcutaneous implants (one of each tumor line). On the day of implantation, samples of each tumor cell line preparation were quantitated for viable cell mass by a stable end point MTT assay so that the time zero cell mass is known. Mice were treated with compound **30** starting on day 4 after fiber implantation and continuing daily for 4 days. The compound was administered by ip injection at two dose levels: 150 and 100.5 mg/kg. The vehicle was 10% DMSO in saline/0.05% Tween 80. The fibers were collected from the mice on the day following the fourth treatment and subjected to the stable end point MTT assay. The optical density of each sample was determined spectrophotometrically at 540 nm, and the mean of each treatment group was calculated. The percent net growth for each cell line in each treatment group was calculated and compared to the percent net growth in the vehicle treated controls. A 50% or greater reduction in percent growth of the treated samples compared to the vehicle control samples was considered a positive result. Each positive result was given a score of 2, and all of the scores were totaled. The maximum possible score for the described experiment is 96 (12 cell lines × 2 sites × 2 dose levels × 2 [score]).

**d. Cell Culture and Treatment.** The human breast adenocarcinoma cell line MCF-7 was maintained in RPMI 1640 medium (Euroclone, Italy), supplemented with 10% FBS (Euroclone, Italy) and 2 mM L-glutamine (Sigma) at 37 °C and 5% $CO_2$. Working solutions of compounds under test were prepared in DMSO at 10 mmol/L immediately before use. Cells were plated at $2 \times 10^4$ cells/cm$^2$ in plastic culture wells, and after 24 h the derivatives were added to the medium to obtain the indicated concentrations. In control cells, DMSO was added to the culture medium. At the end of the treatment, cells were harvested using 0.11% trypsin (Sigma) and 0.02% EDTA (Sigma) and counted. Cell viability was determined by trypan blue exclusion.

**e. Cell Cycle Analysis.** MCF-7 control and treated cells were detached with 0.11% trypsin (Sigma-Aldrich, St. Louis, MO) and 0.02% EDTA, washed twice in PBS, and centrifuged. The pellet was suspended in 0.01% nonidet P-40 (Sigma), 10 $\mu$g/mL RNase, and 0.1% sodium citrate (Sigma), and 50 $\mu$g/mL propidium iodide (PI) (Sigma) for 30 min at room temperature in the dark. Propidium iodide fluorescence was analyzed using a Bryte flow cytometer (Biorad), and cell cycle analysis was performed by means of a Coulter Epics XL MCL cytometer. Cell cycle analysis was performed using Modfit 5.0 software.

**f. Western Blotting.** The cells were collected in 5 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 0.1% Triton X-100, and protease inhibitors in 20 mM HEPES, pH 7.5, and subjected to two cycles of freeze−thawing. The cell proteins in the homogenate were extracted, separated by SDS−PAGE, and detected as described.[36] Primary antibodies against total and phosphorylated ERK 1/2 and Akt were from Cell Signaling Technology, Danvers, MA; anti-p53, anti-p21, and anti-$\beta$-actin antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA. Quantitative assay of immunoblotting was obtained by densitometry with a Fluor-S Max Multilmager instrument (Bio-Rad).

**g. Immunofluorescence Confocal Microscopy.** MCF-7 cells were seeded at $1 \times 10^4$ cells/cm$^2$ on glass coverslips. At the end of the incubation, cells were washed twice and fixed with 3% *p*-formaldehyde, washed with 0.1 M glycine in PBS, and permeabilized in 70% ice-cold ethanol. Bax staining was obtained by incubation for 1 h at room temperature with a mouse monoclonal anti-Bax antibody (BioSource). Cells were then washed, incubated with FITC-coniugated antimouse IgG antibody (Sigma) for 1 h, and finally stained with propidium iodide. All preparations were embedded in Mowiol and analyzed using a Nikon C1s confocal laser-scanning microscope equipped with Nikon eclipse TE300. Multiple images were acquired by using sequential laser excitations at 488 and 568 nm to reduce spectral bleed-through artifacts.

**h. Caspase Activity.** The activity of caspase enzymes hydrolyzing the peptide sequence DEVD, indicated as DEVDase activity, was measured in cell extracts by a fluorometric assay.[36]

**i. Data Analysis.** All the experiments on the effects of studied compounds on MCF-7 cells were performed independently three or four times with comparable results. The results are expressed as mean values ± SEM of the data obtained in the indicated numbers of independent experiments. When statistical analysis was applicable, data were compared by the Student *t* test. Differences were considered significant for $P < 0.05$.

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**Supporting Information Available:** Additional IR and $^1$H NMR spectra, elemental analysis results (Table S1), NSC numbers (Table S2), antitumor activity expressed as the negative log of the molar concentration at three assay end points (Table S3), and mean graph of compound **30** where all the cell lines employed are reported (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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