Antitumor Activity of Substituted *E*-3-(3,4,5-Trimethoxybenzylidene)-1,3-dihydroindol-2-ones[1]

Aldo Andreani,*,† Silvia Burnelli,† Massimiliano Granaiola,† Alberto Leoni,† Alessandra Locatelli,† Rita Morigi,† Mirella Rambaldi,† Lucilla Varoli,† and Mark W. Kunkel‡

Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy, and Developmental Therapeutics Program, Information Technology Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, 6130 Executive Boulevard, Room 8008, Rockville, Maryland 20892-7444

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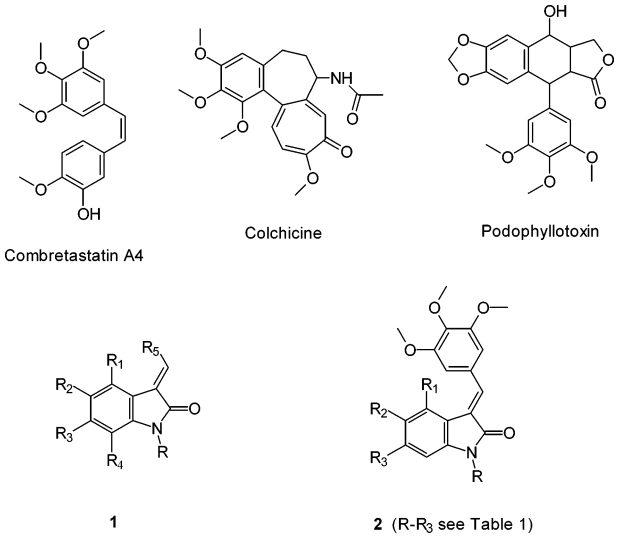
The design and synthesis of anticancer *E*-3-(3,4,5-trimethoxybenzylidene)-1,3-dihydroindol-2-ones is reported. Strong COMPARE correlations among the cell line responses suggest that these compounds may be acting similarly through a combination of different mechanisms of action. The 5-methoxy derivative (**2h**) was the most active compound with a mean pGI_{50} of 6.34, and it is now under review by Biological Evaluation Committee of the National Cancer Institute for possible further studies.

Introduction

We published the pharmacological activity of a large series of compounds related to the general formula **1** (Chart 1) where R_5 represents a monocyclic[2,3] or a bicyclic system. The most recent papers are related to the anticancer activity of this second group of compounds where R_5 is another indole (for the last paper in this series, see ref 4) or an imidazothiazole system (for the last paper in this series, see ref 5). We report now the synthesis and antitumor activity of analogues (**2**) bearing a 3,4,5-trimethoxyphenyl group, since it is present, free, or hindered in well-known antitumor agents such as combretastatin, colchicine, and podophyllotoxin (Chart 1). From a literature survey we saw that two of the compounds we planned (**2a**[6] and **2h**[7]) were already known but their anticancer activity had not been described. Another compound (**2e**) had been reported in the literature, although its activity was not being investigated in relation to cancer.[8] Moreover, the analogue bearing the methoxy group at the 6 instead of the 5 position of the indole system had been studied as an anticancer agent[9] even though on three cell lines only. For a recent review on combretastatin and its analogues see ref 10.

Chart 1



Chemistry

Compounds **2** (see Chart 1 and Table 1) were synthesized by means of the Knoevenagel reaction between 3,4,5-trimethoxybenzaldehyde and the appropriate oxindole. The reaction was performed in methanol in the presence of piperidine (method A). The only exceptions are **2f**−**g**; the first one was prepared in AcOH/HCl (method B) and the second in AcOH/AcONa (method C).

The ^{1}H NMR spectra (Table S1 in Supporting Information) are in agreement with the assigned structures. The above-mentioned authors[9] used a literature reference to establish that their 6-methoxy analogue belongs to the *E* configuration (the aromatic protons of the trimethoxyphenyl ring are in the range 7.45−7.84 ppm). Since the same protons in the *Z* configuration should be in the range 7.85−8.53 ppm, we do not believe that the two groups of chemical shifts are significantly different. Moreover, the compounds described in the considered reference are not trimethoxyphenyl derivatives. Therefore, we selected compound **2h** to perform a NOE experiment. The *E* configuration was confirmed by the irradiation of the methine bridge (7.58 ppm), which gave NOE at 7.06 ppm (the aromatic protons of the trimethoxyphenyl ring) and not at the H-4 of the indolinone system (7.26 ppm).

Table 1. Compounds **2a**−**i**

compd	R	R_1	R_2	R_3	formula	MW
2a	H	H	H	H	$C_{18}H_{17}NO_4$	311.33
2b	CH_3	H	H	H	$C_{19}H_{19}NO_4$	325.36
2c	C_6H_5	H	H	H	$C_{24}H_{21}NO_4$	387.43
2d	H	Cl	H	H	$C_{18}H_{16}ClNO_4$	345.78
2e	H	H	Cl	H	$C_{18}H_{16}ClNO_4$	345.78
2f	H	H	F	H	$C_{18}H_{16}FNO_4$	329.32
2g	H	H	OH	H	$C_{18}H_{17}NO_5$	327.33
2h	H	H	OCH_3	H	$C_{19}H_{19}NO_5$	341.36
2i	H	H	OCH_3	CH_3	$C_{20}H_{21}NO_5$	355.39

All compounds **2** were obtained as pure geometrical isomers, but their stability in DMSO-d_6 is not the same for all the compounds. For example, **2e** and **2g** were mixtures even in a freshly prepared solution. Only four compounds were recorded after at least 24 h; **2d** and **2e** were stable, whereas **2c** and **2i** were *E*/*Z* mixtures. A systematic study of this issue has not been performed, but we plan to evaluate this effect in forthcoming papers because the possible influence on the biological activity is obvious.

* To whom correspondence should be addressed. Phone and fax: +39-(0)51-2099714. E-mail aldo.andreani@unibo.it.
† Università di Bologna.
‡ National Cancer Institute.

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Table 2. Growth Inhibition and Cytostatic and Cytotoxic Activity of Compounds **2a**−**i** in the 60-Cell Panel

compd[a]	modes	leukemia	NSCLC	colon	CNS	melanoma	ovarian	renal	prostate	breast	MG-MID[b]
2a	pGI_{50}	5.12	4.73	4.82	4.91	4.70	4.61	4.73	4.73	4.72	4.78
	pTGI	4.06	4.07	4.12	4.07	4.09	4.02	4.12		4.11	4.08
2b	pGI_{50}	5.14	4.74	4.87	5.02	4.76	4.74	4.91	4.98	4.71	4.85
	pTGI	4.15	4.17	4.21	4.09	4.36	4.09	4.21		4.11	4.18
2c	pGI_{50}	5.64	5.03	5.24	5.22	5.13	5.00	5.24	5.50	5.08	5.19
	pTGI	4.85	4.44	4.57	4.51	4.68	4.49	4.63	4.79	4.27	4.56
	pLC_{50}	4.01	4.08	4.17	4.14	4.28	4.08	4.19			4.12
2d	pGI_{50}	5.62	4.12		4.21		4.05	4.21		4.07	4.25
	pTGI	4.79			4.01						4.09
2e	pGI_{50}	5.06	4.68	5.08	4.76	4.55	4.77	4.80	5.53	4.80	4.82
	pTGI		4.13	4.03	4.25		4.11	4.19		4.25	4.12
2f	pGI_{50}	4.85	4.57	4.51	4.48	4.43	4.35	4.46	4.56	5.02	4.58
	pTGI		4.04		4.07					4.06	4.02
2g	pGI_{50}	5.52	4.93	4.91	5.06	5.00	4.85	4.85	5.62	5.04	5.02
	pTGI	4.51	4.39	4.32	4.38	4.56	4.32	4.35	4.72	4.28	4.40
	pLC_{50}		4.05	4.08	4.04	4.16	4.03	4.08		4.01	4.06
2h[c]	pGI_{50}	7.06	5.95	6.74	6.38	6.05	6.17	6.24	6.16	6.40	6.34
	pTGI	4.95	4.23	4.49	4.90	4.38	4.81	4.72		4.83	4.61
	pLC_{50}			4.08	4.02	4.02	4.38	4.04		4.30	4.09
2i	pGI_{50}	4.74	4.24	4.07	4.38	4.13	4.12	4.26		4.28	4.26
	pTGI	4.28	4.09		4.08			4.05		4.03	4.06
vin[d]	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 concentration is 10^{-4} M unless otherwise reported. Only modes showing a value >4.00 are reported, i.e., only concentrations lower than 10^{-4} M. [b] Mean Graph MIDpoint, i.e., the calculated mean panel. [c] Mean of two separate experiments [d] Vincristine sulfate. Highest concentration is 10^{-3} M according to the NCI Standard Agents Database, http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent_table.html.

Biology

(a) Anticancer Activity. As a primary screening, **2a**−**i** were submitted to the National Cancer Institute (NCI) cell line screen for evaluation of their anticancer activity. In a preliminary test at a single concentration (100 μM) 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. By these criteria, all of the compounds reported in Table 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 and the others being 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M. Table 2 reports the results obtained with this test (vincristine is reported for comparison purposes) expressed as the −log of the molar concentration that inhibited cell growth by 50% (pGI_{50}), that caused total cytostasis (pTGI, total growth inhibition), or that killed half of the cells (pLC_{50}).

The assay of **2h** was repeated once; other compounds were assayed once. At the pGI_{50} endpoint the analogues were of approximately equal overall potency; the average of the log molar concentrations for all cell lines ranged from 4.2 to 5.2 compared to the average potency of **2h**, which was 6.3. The range between the least sensitive and most sensitive cell lines (data not shown) was 4 log units for **2h**. The range between the least sensitive and the most sensitive cell lines was smaller for the other compounds, 1−2 log units. At the pTGI endpoint the average log molar potencies of the analogues were tighter, ranging from 4.0 to 4.6. The average potency for **2h** was 4.6. The range between the least sensitive and the most sensitive cell lines was roughly comparable to the ranges seen at the pGI_{50} endpoint, though the overall patterns showed less difference among the cell lines.

(b) COMPARE. We used the COMPARE algorithm to look at the overall patterns of responsiveness of the cell lines. Strong COMPARE correlations (empirically defined as correlations greater than 0.5) among the cell line responses suggest that compounds may be acting through similar mechanisms[11] whose nature is still to be determined. We focused on the GI_{50} endpoint because the TGI data showed less overall discrimination among the cell lines. At the GI_{50} endpoint (Table S2 in Supporting Information), the pairwise correlations among four of the compounds (**2a**−**c**,**g**) suggested that they were acting via similar mechanisms. Compounds **2d** and **2i** were strongly correlated and showed a moderate correlation to the other compounds through the correlations linking **2d** with **2c** and **2g**.

We also used the COMPARE algorithm to search the NCI database of public compounds and public molecular targets for compounds and molecular targets with activity or expression patterns that correlated with the activity patterns of **2a**−**c**,**g**. Table S3 lists compounds whose GI_{50} activity pattern across the cell lines correlated positively with at least three of the four analogues; there are an additional 60 compounds that were common to at least two of the four analogues (not shown). The structures (when known) of the compounds can be accessed at http://dtp.nci.nih.gov/dtpstandard/ChemData/index.jsp.

A few of these compounds are discussed in the chemical literature, but none of them have a known mechanism of action. Of interest is that none of the known tubulin interacting agents that might be expected to correlate with these analogues[9] were in the set of compounds identified by COMPARE; however, there are also no apparent structural similarities between the analogues and the compounds identified by COMPARE. Table S4 lists molecular targets and Table S5 lists microarray targets whose expression patterns across the cell lines correlated positively or negatively (see Experimental Section) with the analogues. Resolution of this list to the KEGG, BIOCARTA, and GO databases shows some common members of pathways for signal transduction and the control and execution of cell division.

Conclusion

The 5-methoxy derivative (**2h**) was the most active compound with a mean pGI_{50} of 6.34. Its potency was similar to vincristine,

and the difference between the average concentration that caused 50% growth inhibition and the concentration that killed 50% of the cells was 2.46 log. It is now under review by Biological Evaluation Committee of the NCI for possible further studies.

Even though this series is formed by only eight members, the importance of the substituents at the nitrogen (**2a**−**c**; the anticancer activity of the phenyl derivative **2c** is noteworthy) and at the indole benzene ring (**2d**−**i**) is evident. In this set of compounds, besides the above 5-methoxy derivative **2h**, the 5-hydroxy analogue **2g** deserves mention too.

Our first paper on indolinone derivatives connected to another cyclic system by means of a methine bridge is dated 1996.[12] Testing several members of this class of compounds against different targets and usually finding an activity that is moderate but never strong in only one of them make us suppose that these compounds act by means of multiple mechanisms, and the results from COMPARE support this hypothesis. The theory of compounds acting by means of multiple mechanisms is not new. Sosinski et al.[13] suggested the participation of two different mechanisms of action in a series of diarylsulfonylureas. Xiang et al.[14] demonstrated that protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. ZR2002 is a chimeric aminoquinazoline designed to possess mixed EGFR tyrosine kinase and DNA targeting properties.[15] The multiple mechanisms of action of the flavonoid casticin, derived from *Achillea millefolium*, were recently described.[16] It gives rise to cell growth arrest in G2/M and apoptotic death. As a tubulin-binding agent, it induces p21, which in turn inhibits Cdk1. In addition, it appears to down-regulate cyclin A.

We also assume that several antitumor agents published with a univocal mechanism of action could also display activity against additional and still unexplored targets.

Experimental Section

(1) Chemistry. Synthesis of the 3-(3,4,5-Trimethoxybenzylidene)-1,3-dihydroindol-2-ones. Method A (2a-e,h,i). 3,4,5-Trimethoxybenzaldehyde (10 mmol) was dissolved in methanol (100 mL) and treated with the equivalent of the appropriate indolinone 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 (yield 45−55% for **2c**,**i**; 80−90% for the others) and crystallized from methanol.

Method B (2f). 3,4,5-Trimethoxybenzaldehyde (10 mmol) was dissolved in acetic acid (50 mL) and treated with the equivalent of 5-fluoro-2-indolinone and 37% hydrochloric acid (1 mL). The reaction mixture was refluxed for 4 h, and the precipitate formed on cooling was collected by filtration (yield 40%) and crystallized from methanol.

Method C (2g). 3,4,5-Trimethoxybenzaldehyde (15 mmol) was treated with the equivalent of 5-hydroxy-2-indolinone, 30 mmol of anhydrous sodium acetate, and 130 mL of acetic acid. The reaction mixture was refluxed for 3 h, evaporated under reduced pressure, and poured into ice−water. The resulting precipitate was collected by filtration (yield 60%) and crystallized from methanol.

(2) Biology. The anticancer tests were performed by the NCI (Bethesda, MD) as in our previous papers.

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Supporting Information Available: Detailed experimental procedures with analytical and spectroscopic data for all the compounds; tables of data from use of COMPARE. This material is available free of charge via the Internet at http://pubs.acs.org.

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