Activation of Human Telomerase Reverse Transcriptase Expression by Some New Symmetrical Bis-Substituted Derivatives of the Anthraquinone

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As a part of our program aimed at exploring the biological activity of symmetrical substitution of side chains into the anthracene-9,10-dione chromophore, we have synthesized a series of 1,5-bisthioanthraquinones **2** and 1,5-bisacyloxyanthraquinones **3** that are related to the antitumor agent mitoxantrone. Since the telomerase enzyme is a novel target for potential anticancer therapy and stem cell expansion, we explore the biological effects of these compounds by evaluating their effects on telomerase activity and telomerase expression. Telomerase is required for telomere maintenance and is active in most human cancers and in germinal cells but not in most of the normal human somatic tissues. We found that most of the 1,5-disubstituted anthraquinones did not exhibit inhibitory activity at the concentration ranging from 20 to 30 μM. To facilitate the analysis of the expression of telomerase, we used cancer and normal cell lines that carry secreted alkaline phosphatase (SEAP) gene under the control of human telomerase reverse transcriptase (hTERT). The effects of these compounds on the expression of telomerease were analyzed using the cell-based reporter systems. While most of these compounds did not appear to selectively repress the expression of hTERT in cancer cells, compounds **3a**, **3d**, and **3i** activated hTERT expression in normal cells. The effects of these three compounds on hTERT expression appear to be specific because they did not increase the expression of a CMV promoter-driven SEAP. Thus, in addition to anticancer functions, our finding raises the possibility that these compounds might also have a role in cell immortilization. The application of these anthraquinone derivatives in stem cell research and tissue engineering is also discussed.

Introduction

Telomerase is a ribonucleoprotein that contains both RNA and protein component for the maintenance of telomere length. Telomerase activation in some cell types appears to offset proliferation-dependent telomere shortening, which delays the inherent mitotic clock.[1] This enzyme comprises a template-containing RNA subunit, hTR (human telomerase RNA component), and catalytic protein subunit hTERT (human telomerase reverse transcriptase) in human. In most normal human cells, telomerase activity is low or not detectable because telomeric DNA is progressively shortened with each cell division. It is hypothesized that progressive shortening of telomeres eventually causes cellular senescence. This is supported by the immortalization of normal human cells with the introduction of hTERT gene into these cells.[2−5] Moreover, telomerase activity was detected in germinal cells, immortalized cell lines, and in 85−90% of human cancer specimens, further supporting the role of telomerase in cell immortalization and in cancer progression. The presence of telomerase activity in tumors combined with its absence in most normal tissues has generated considerable interest in using telomerase inhibitors as cancer therapeutic agents. Recently, telomerase activation was considered as a means for the expansion of somatic stem cells. Indeed, several studies have already investigated adrenocortical cells,[6] endothelial cells,[7] and bone marrow stromal stem cells in vitro by introducting hTERT into these cells and restoring the cellular function.[8,9] The expression of TERT, the reverse transcriptase component of telomerase, is necessary to convert normal human cells into cancer cells.[10] Thus, inhibition or activation of the reverse transcriptase telomerase can profoundly affect the proliferative capacity of normal cells and cancers.[11]

The hTERT expression appears to be the key regulator for telomerase activity. The expression of hTERT is regulated by several factors, and recent interest in the regulation of telomerase, the enzyme that maintains chromosomal termini, has led to the discovery and characterization of the catalytic subunit of hTERT.[12] For example, expression of c-Myc was shown to induce the expression of hTERT, whereas the expression of Sp1 (stimulating protein 1) suppresses it. However, it remains unclear how hTERT is inactivated during development and how it is reactivated during tumorigenesis. Research into the regulation of telomerase may lead to the development of methods for accurate diagnosis of cancer and to the development of novel antitelomerase cancer therapeutics.[13−16] Consequently, telomerase be-

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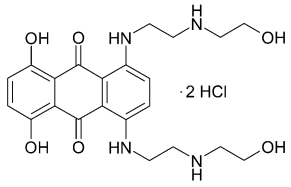
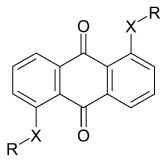
Chart 1. Structures of Mitoxantrone and Anthraquinone Derivatives

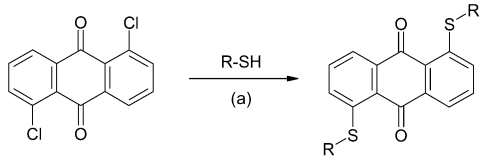


1 Mitoxantrone dihydrochloride



2 X = S; **3** X = O

Scheme 1[a]



2a-o

[a] Reagents: (a) sodium methoxide/methanol, tetrahydrofuran, reflux.

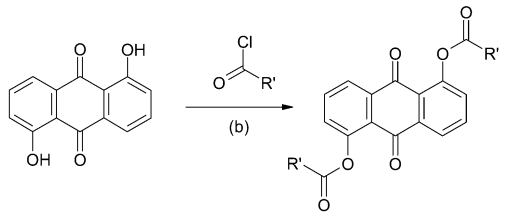
came an obligate focus for anticancer research because it promised a unique target for chemotherapy without the associated side effects of conventional chemotherapeutics.[17]

Anthraquinone-based compounds currently occupy a prominent position in cancer chemotherapy, and the anthraquinone mitoxantrone (**1**, Chart 1) is an important compound used clinically as an anticancer agent.[18] It has been used in treating human leukemia, lymphomas, and advanced breast and ovarian cancers in combination with other treatments.[19] Although the reaction mechanism of the antitumor activity of anthraquinone is probably multimodal in nature, studies have indicated that its intercalative interaction with DNA may play a major role.[20−22] Human telomerase has been proposed as a highly selective target for antitumor drug design.[23,24] This hypothesis is supported by experiments with antisense constructs against telomerase RNA in HeLa cells, which show that telomere length is progressively shortened, together with the death of these otherwise immortal cells.[25] From the studies on the structure−activity relationships of anthraquinones, we have recently reported the synthesis of a number of 1,5-bisthioanthraquinones and 1,5-bisacyloxyanthraquinones (**2**, **3**; Chart 1) that exhibited potent cytotoxicity against rat glioma C6 cells, human hepatoma G2 cells, and lipid peroxidation compared to mitoxantrone.[26,27] Here, we present the effects of this new class of compounds on a biologically important enzyme telomerase. We also examine the effects of these compounds on hTERT expression using cell-based assay systems. We found that several of these bis-substituted anthraquinone analogues activate hTERT expression. Our results suggest that the compounds with anthraquinone moieties could have a role in cell immortalization that was not described before. Side chains have been examined to establish structure−activity relationships as a basis for subsequent rational drug design.

Chemistry

The synthesis of the target compounds is illustrated in Schemes 1 and 2. Various side chains are attached to anthraquinone using electrophilic additions or substitutions. Anthraquinone may be considered to consist of two benzoyl chromogens in which little or no interaction would be expected between substituents located in different benzene rings. In Scheme 1, the synthesis of 1,5-bisthioanthraquinones was carried out by a nucleophilic substitution at the 1 and 5 positions with the appropriate thiols in the presence of sodium methoxide and THF at room temperature or after refluxing for 1−2 h. In these reactions, sodium methoxide ionized the thiols that could then serve as nucleophiles for nucleophilic substitutions. The synthesis of 1,5-bisacyloxyanthraquinones shown in Scheme 2 was based on simple acylation involving 1,5-dihydroxyanthraquinone (anthrarufin) with an excess of the appropriate acyl chlorides in the presence of pyridine and dichloromethane at room temperature for 1−2 h or in the presence of NaH and THF at room temperature or after refluxing for 1−2 h. All final products were characterized by NMR, IR, UV, and mass spectrometry.

Scheme 2[a]



3a-n

[a] Reagents: (b) method A, pyridine; method B, NaH, THF.

Biological Results and Discussion

The chemical and biological activities of anthraquinone compounds are greatly affected by various substituents of the planar ring system.[28−35] We have previously examined the cytotoxic activity in several tumor cell lines and their inhibitory effects on lipid peroxidation. Since telomerase is an important component of cancers, we are interested in examining the effects of these compounds on telomerase activity in the cell-free extracts prepared from H1299 cells. We applied a PCR-based telomerase assay, TRAP (telomeric repeat amplification protocol) assay, as a system for assaying the telomerase activity. The inhibitory effect of anthraquinones at 20−30 μM concentrations was tested. We reasoned that an effective telomerase inhibitor should give good inhibitory effect at this concentration. Among a total of more than 30 pure compounds tested, none of them exhibited significant telomerase inhibitory activity (data not shown). According to our previous study on 9-acyloxy-1,5-dichloroanthracene derivatives, we did not observe any specific telomerase inhibitory activity.[36] It is interesting to note that even though the compounds we synthesized do not inhibit telomerase, a report on amino-disubstituted anthracenc derivatives indicates that some of these compounds inhibit telomerase.[37]

Because telomerase expression is a hallmark of cancer, the effect of anthraquinones on telomerase expression was determined. The telomerase activity is regulated mostly at the transcriptional level for its catalytic subunit, hTERT, and partly at the posttranslational level.[38] Since the expression of human telomerase catalytic component is the key regulator in

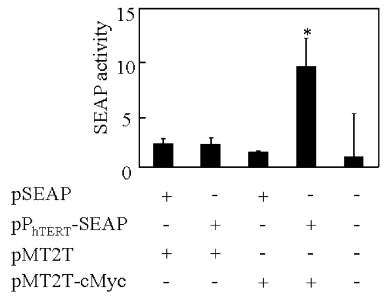


Figure 1. Activation of hTERT promoter-driven SEAP expression by c-Myc. About 1×10^7 hTERT-BJ1 cells were transfected with 13.5 μg each of plasmid pSEAP or pPhTERT-SEAP and of plasmid pMT2T or pMT2T-cMyc by electroporation. After 24 h, viable cells were harvested and reinoculated at a density of 3×10^5/mL, and the SEAP activity was determined after 24 h at 37 °C. The transfection efficiency of each experiment was determined by cotransfection with 1.5 μg of plasmid pCMVβ. The values were determined from three experiments. $P < 0.05$ is presented by an asterisk.

telomerase activity, we analyzed the expression of telomerase by monitoring the expression of hTERT as the criteria. To facilitate the analysis, we ligated downstream to the hTERT promoter a reporter gene, secreted alkaline phosphatase (SEAP), to generate the hTERT promoter-driven reporter construct, P$_{hTERT}$-SEAP. In this construct, the 3.4 kbp hTERT promoter region ranging from position −3338 to +1 was used. This DNA fragment contains the cis regulatory elements of hTERT transcription. To test if our reporter is functional, the SEAP expression was monitored in the presence of c-Myc because it was shown to be a positive regulator of hTERT. As shown in Figure 1, the SEAP activity is elevated in cells that are cotransfected with P$_{hTERT}$-SEAP and plasmid that expresses c-Myc. This result indicated that our reporter construct is capable of activating the expression of SEAP activity.

Plasmid construct pP$_{hTERT}$-SEAP or a CMV (cytomegalovirus) promoter-driven SEAP construct, pP$_{CMV}$-SEAP were introduced into cancer cell line H1299 and hTERT-immortalized normal cell line hTERT-BJ1. Stable cell lines harboring P$_{hTERT}$-SEAP or P$_{CMV}$-SEAP were then isolated. We selected one cell line from each of these stable lines for further characterization. We first examined the global phenotype of these cells. Integration of pP$_{hTERT}$-SEAP or pP$_{CMV}$-SEAP did not appear to affect the morphology and the growth rate of their parental cell H1299 or hTERT-BJ1 (Figure 2 and data not shown). Since telomerase is active in cancer cells and inactive in normal cells, we determine the expression of SEAP in these stable cell lines. As shown in Figure 3, H1299 cells harboring P$_{hTERT}$-SEAP expressed a high level of SEAP activity, whereas hTERT-BJ1 cells harboring P$_{hTERT}$-SEAP only expressed low-to-zero level of SEAP activity. These results indicate that the 3.4 kbp DNA fragment used in our study is sufficient to confer the properties of hTERT promoter. Moreover, our results demonstrate that cells harboring P$_{hTERT}$-SEAP could be used as a tool to monitor the expression of hTERT.

The expression of SEAP in H1299 cells harboring P$_{hTERT}$-SEAP was used as the criterion to evaluate if anthraquinone derivatives inhibited the expression of hTERT in cancer cells. The level of cell viability in these cells was also determined using MTT assay. As shown

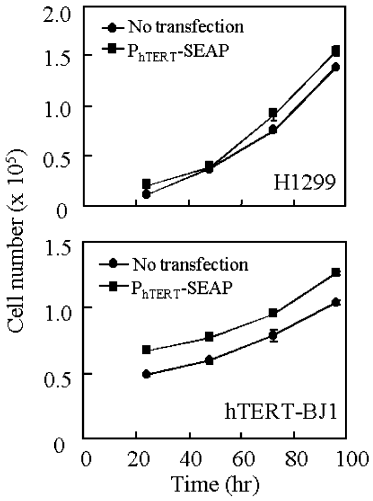


Figure 2. Stable cell lines harboring P$_{hTERT}$-SEAP did not affect the growth rate of their parental cell lines. H1299, hTERT-BJ1, and stable cell lines harboring P$_{hTERT}$-SEAP were grown at 37 °C in the presence of 5% CO_2. The cell growth was monitored for a period of 96 h using MTT assay. The values are determined from four experiments.

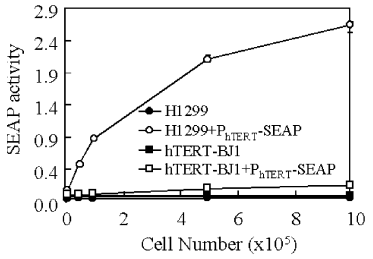
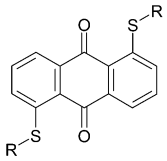


Figure 3. Expression of P$_{hTERT}$-SEAP in H1299 cells and lack of P$_{hTERT}$-SEAP expression in hTERT-BJ1 cells. H1299, hTERT-BJ1, and stable cell lines harboring P$_{hTERT}$-SEAP from these two cells were analyzed for SEAP activity. The values are determined from three experiments.

in Tables 1 and 2, most of the compounds did not show significant effects on SEAP expression. Only compounds **2b**, **2c**, **2f**, **2l**, **3a**, and **3n** affected the SEAP expression. These six compounds also affect the proliferation of the treated cells. Thus, although it is not apparent whether the telomerase repression effects of these compounds are caused by their cytotoxicity, it is conceivable that inhibition of telomerase expression by these compounds inhibit cell proliferation.

We then used the expression of SEAP in hTERT-BJ1 cells harboring P$_{hTERT}$-SEAP as the criterion to evaluate if anthraquinones activate the expression hTERT in normal cells. The level of cell viability in these cells was also determined using MTT assay, and the results are shown in Tables 1 and 2. Interestingly, cytotoxicity levels in human tumor cell lines were at comparable levels for several compounds; nearly half of the anthraquinone derivatives activated the SEAP levels. Among these compounds, we are most interested in compounds **3a**, **3d**, and **3i** because these compounds activated the SEAP levels without affecting the cell viability (Figure 4). To test if these three compounds affect the expression of the other promoter, we analyzed the effects of these three compounds on the activity of the CMV promoter. Here, we introduce a CMV promoter-driven SEAP, P$_{CMV}$-SEAP, into hTERT-BJ1 to generate stable cell lines and used these cell lines for analysis.

Table 1. Effects of Symmetrical 1,5-Bisthio-Substituted Anthraquinones (**2a**−**o**) on Respressing and Activating hTERT Expression



2a-o

			P_{hTERT}-SEAP (H1299)[b]		P_{hTERT}-SEAP (hTERT-BJ1)[c]	
compd	R	concn[a] (μM)	relative viability (%)	relative SEAP activity (%)	relative viability (%)	relative SEAP activity (%)
2a	CH_2CH_3	3.0	111 ± 2.8	134 ± 14.4	112 ± 9.2	109 ± 22.4
		30	44 ± 7.3	111 ± 7.7	98 ± 12.3	110 ± 14.3
		300	25 ± 2.3	99 ± 14.3	34 ± 18.1	104 ± 20.6
2b	CH_2CH_2OH	2.8	54 ± 4.0	97 ± 15.7	94 ± 9.3	104 ± 15.7
		28	29 ± 7.0	76 ± 12.4	49 ± 2.9	98 ± 10.9
		280	36 ± 7.2	45 ± 2.7	23 ± 2.9	71 ± 5.0
2c	$CH_2CH_2CH_3$	2.8	96 ± 5.9	73 ± 5.4	103 ± 6.3	132 ± 21.0
		28	44 ± 2.5	29 ± 2.5	97 ± 3.0	110 ± 13.2
		280	25 ± 2.4	17 ± 13.9	39 ± 4.9	122 ± 14.3
2d	$CH_2CH(OH)CH_2OH$	2.4	102 ± 6.2	105 ± 21.6	98 ± 10.7	144 ± 16.9
		24	103 ± 4.2	90 ± 5.7	86 ± 5.5	136 ± 10.0
		240	83 ± 18.2	81 ± 6.1	79 ± 8.2	142 ± 9.1
2e	$(CH_2)_6OH$	2.1	99 ± 6.2	110 ± 6.1	99 ± 6.2	140 ± 9.7
		21	94 ± 3.9	100 ± 5.5	70 ± 2.2	128 ± 14.4
		210	36 ± 4.2	68 ± 5.9	40 ± 4.7	75 ± 17.4
2f	$2\text{-}NH_2C_6H_4$	2.2	94 ± 3.5	108 ± 12.2	103 ± 7.9	136 ± 17.5
		22	50 ± 3.3	96 ± 8.4	107 ± 5.5	141 ± 18.2
		220	14 ± 2.5	59 ± 6.6	25 ± 3.3	115 ± 29.7
2g	$3\text{-}NH_2C_6H_4$	2.2	92 ± 3.5	106 ± 7.6	104 ± 5.1	118 ± 9.9
		22	68 ± 1.9	109 ± 11.7	9 ± 3.4	41 ± 9.5
		220	32 ± 4.9	101 ± 7.3	4 ± 1.5	8 ± 30.6
2h	$4\text{-}NH_2C_6H_4$	2.2	103 ± 4.3	100 ± 5.7	86 ± 11.9	97 ± 17.9
		22	76 ± 4.0	95 ± 2.6	65 ± 12.6	97 ± 14.5
		220	42 ± 2.3	84 ± 5.3	56 ± 13.8	26 ± 12.6
2i	$CH_2C_6H_5$	2.2	83 ± 5.5	97 ± 6.0	121 ± 4.7	117 ± 11.3
		22	44 ± 0.9	100 ± 7.2	98 ± 4.2	112 ± 9.3
		220	34 ± 3.3	100 ± 12.9	47 ± 9.1	87 ± 11.1
2j	$CH_2C_6H_4(OCH_3)(p)$	2.2	89 ± 6.1	92 ± 3.3	119 ± 9.4	142 ± 27.7
		22	59 ± 5.4	96 ± 8.5	98 ± 13.4	141 ± 22.6
		220	42 ± .3	88 ± 5.6	62 ± 6.4	118 ± 19.2
2k	$CH_2CH_2C_6H_5$	2.2	93 ± 6.2	108 ± 0.5	91 ± 3.9	119 ± 12.2
		22	51 ± 9.3	102 ± 0.5	54 ± 4.4	109 ± 23.4
		220	27 ± 2.9	97 ± 4.8	35 ± 4.1	110 ± 30.6
2l	$C_4H_3N_2$	2.3	107 ± 5.1	105 ± 6.6	104 ± 8.9	137 ± 12.3
		23	99 ± 5.8	110 ± 8.0	103 ± 6.4	125 ± 6.9
		230	44 ± 6.3	49 ± 10.0	73 ± 8.3	61 ± 8.7
2m	C_5H_4N	2.3	79 ± 12.2	104 ± 12.6	112 ± 6.4	98 ± 12.3
		23	45 ± 7.1	103 ± 15.8	91 ± 7.3	133 ± 6.8
		230	29 ± 1.5	89 ± 9.3	55 ± 11.3	121 ± 13.8
2n	$C_4H_2N_2(OH)(m)$	2.2	99 ± 4.0	92 ± 7.1	104 ± 7.6	93 ± 7.9
		22	95 ± 4.9	97 ± 2.9	106 ± 6.3	116 ± 15.4
		220	51 ± 16.7	93 ± 12.1	89 ± 3.5	145 ± 20.7
2o	$C_6H_4CH_3$	2.2	84 ± 5.9	117 ± 12.5	111 ± 5.4	132 ± 19.3
		22	41 ± 2.4	103 ± 10.5	77 ± 6.9	109 ± 5.7
		220	25 ± 2.4	90 ± 11.6	22 ± 8.5	87 ± 29.6

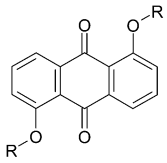
[a] Values are in μM and represent an average of three experiments. The variance for the relative viability (%) and relative SEAP activity (%) values was less than ±20%. Activity of P_{hTERT}-SEAP (H1299) and (hTERT-BJ1) cell growth was significantly different with respect to that of the control; $n = 3$ or more, $P < 0.01$. Relative percentage of inhibition was not compared with that of the control, $P < 0.01$, mean ± SE, $n = 4$. Values are the mean percent activity at the indicated concentration and include standard errors. [b] Non-small-cell lung cancer cells H1299. [c] The hTERT-immortalized hTERT-BJ1 was purchased from BD Biosciences Clontech.

As shown in Table 3 and Figure 4, none of these three compounds affected the expression of the CMV promoter, suggesting that the effects of these three compounds on hTERT promoter are specific.

Conclusions

Normal human cells undergo a finite number of cell divisions and ultimately enter a nondividing state called replication senescence. It has been proposed that telomere shortening is the molecular clock that triggers senescence.[39] Since telomerase extends the life span of in vitro cultured cells, it has been hypothesized that telomerase therapy may delay age-associated tissue degeneration or reverse organ failure in chronic high-turnover diseases.[40,41] However, since telomerase also has an important role in cancer, it is possible that hTERT transgene expression might be carcinogenic. Indeed, neoplastic lesions are developed in the mammary gland of transgenic mice with TERT expression, suggesting that constitutive telomerase expression might be tumorigenic.[42] Moreover, the risk of genetic alteration upon hTERT integration into chromosome is unknown.

Table 2. Effects of Symmetrical 1,5-Bisacyloxyanthraquinones (**3a**−**n**) on Respressing and Activating hTERT Expression



3a-n

			P_{hTERT}-SEAP (H1299)[b]		P_{hTERT}-SEAP (hTERT-BJ1)[c]	
compd	R	concn[a] (μM)	relative viability (%)	relative SEAP activity (%)	relative viability (%)	relative SEAP activity (%)
3a	$COCH_2CH_3$	2.8	116 ± 8.0	103 ± 5.8	99 ± 11.9	131 ± 7.0
		28	116 ± 7.4	107 ± 4.9	109 ± 8.7	152 ± 16.7
		280	87 ± 6.6	97 ± 3.1	93 ± 10.8	161 ± 12.2
3b	$COCH_2CH_2CH_3$	2.6	107 ± 5.6	114 ± 7.9	109 ± 5.1	107 ± 11.9
		26	106 ± 3.2	118 ± 8.4	106 ± 5.6	119 ± 8.3
		260	87 ± 6.5	111 ± 4.4	85 ± 6.6	134 ± 3.8
3c	$CO(CH_2)_4CH_3$	2.3	117 ± 5.1	91 ± 9.9	119 ± 9.4	147 ± 18.8
		23	114 ± 4.7	95 ± 17.4	98 ± 13.4	149 ± 16.1
		230	106 ± 4.3	95 ± 10.5	62 ± 6.4	124 ± 5.3
3d	$COC(CH_3)_3$	2.4	106 ± 8.9	105 ± 4.9	94 ± 3.4	147 ± 11.1
		24	97 ± 7.1	78 ± 14.8	93 ± 6.0	165 ± 18.7
		240	70 ± 5.2	80 ± 10.1	100 ± 9.0	141 ± 14.4
3e	COC_6H_5	2.2	99 ± 7.3	88 ± 10.0	103 ± 6.5	137 ± 10.0
		22	60 ± 11.9	94 ± 8.0	87 ± 9.8	122 ± 9.6
		220	33 ± 4.7	86 ± 4.7	51 ± 4.6	84 ± 21.7
3f	$COC_6H_4Cl(o)$	1.9	74 ± 5.3	93 ± 4.4	107 ± 5.5	120 ± 10.5
		19	34 ± 3.4	101 ± 4.9	92 ± 4.3	116 ± 3.7
		190	30 ± 1.4	97 ± 5.3	40 ± 4.0	97 ± 10.8
3g	$COC_6H_4Cl(m)$	1.9	98 ± 3.9	83 ± 5.7	101 ± 2.3	154 ± 23.0
		19	88 ± 7.2	92 ± 8.1	80 ± 6.7	152 ± 15.8
		190	46 ± 3.5	81 ± 2.5	44 ± 6.0	91 ± 25.2
3h	$COC_6H_4Cl(p)$	1.9	91 ± 10.0	106 ± 4.5	111 ± 6.5	136 ± 5.7
		19	57 ± 1.8	106 ± 4.9	89 ± 14.3	123 ± 6.2
		190	31 ± 1.0	97 ± 4.9	48 ± 9.0	107 ± 6.0
3i	$COC_6H_4Cl_2(o,p)$	1.8	108 ± 3.8	100 ± 5.2	107 ± 7.2	155 ± 16.3
		18	103 ± 5.9	102 ± 5.4	102 ± 4.1	150 ± 13.9
		180	77 ± 2.8	96 ± 4.4	96 ± 7.0	160 ± 36.7
3j	$COC_6H_4CH_3(o)$	2.1	72 ± 4.9	97 ± 4.4	118 ± 11.6	129 ± 13.0
		21	36 ± 9.7	91 ± 7.4	82 ± 8.5	120 ± 24.0
		210	45 ± 8.0	90 ± 4.2	39 ± 13.0	94 ± 27.1
3k	$COC_6H_4CH_3(m)$	2.1	26 ± 3.7	104 ± 5.4	98 ± 9.4	141 ± 10.5
		21	28 ± 5.0	116 ± 12.8	54 ± 7.5	124 ± 15.6
		210	29 ± 3.3	110 ± 16.4	47 ± 4.8	86 ± 17.2
3l	$COC_6H_4CH_3(p)$	2.1	61 ± 5.8	98 ± 1.1	102 ± 13.9	130 ± 8.8
		21	33 ± 2.8	95 ± 4.3	98 ± 6.3	126 ± 13.3
		210	32 ± 5.7	95 ± 8.9	56 ± 6.2	99 ± 15.8
3m	$COCH_2C_6H_5$	2.1	91 ± 4.2	98 ± 0.3	106 ± 9.0	129 ± 4.5
		21	53 ± 2.3	101 ± 6.8	97 ± 8.2	125 ± 4.3
		210	30 ± 1.6	142 ± 14	64 ± 10.2	100 ± 17.3
3n	$COCH_2CH_2C_6H_5$	2.0	111 ± 0.8	94 ± 2.5	111 ± 7.3	126 ± 6.1
		20	101 ± 4.3	98 ± 4.7	106 ± 6.6	124 ± 13.4
		200	54 ± 4.1	89 ± 5.2	76 ± 7.2	110 ± 10.5

[a] Values are in μM and represent an average of three experiments. The variance for the relative viability (%) and relative SEAP activity (%) values was less than ±20%. Activity of P_{hTERT}-SEAP (H1299) and (hTERT-BJ1) cell growth was significantly different from that of the control; $n = 3$ or more, $P < 0.01$. Relative percentage of inhibition was not compared with that of the control, $P < 0.01$, mean ± SE, $n = 4$. Values are the mean percent activity at the indicated concentration and include standard errors. [b] Non-small-cell lung cancer cells H1299. [c] The hTERT immortalized hTERT-BJ1 was purchased from BD Biosciences Clontech.

We report here the identification of small-molecule compounds that activate hTERT expression without going through procedures that cause genetic alteration in cells. The telomerase activating compounds should be valuable in the fields of stem cell and tissue engineering research in expending target cells. They may also be applied in treating age-associated tissue degeneration or reverse organ failure in chronic high-turnover diseases. This unique property should definitely be noted in future drug design.

The aim of this study was to elucidate the structure−activity relationships of simple 1,5-symmetrical bis-substituted analogues of anthraquinone to further delineate the nature of the requirements of the pharmacophore. A striking feature of the results given in Tables 1−3 are the marked repressing hTERT expression [P_{hTERT}-SEAP (H1299)] and activating hTERT expression [P_{hTERT}-SEAP (hTERT-BJ1)]. Overall, compounds **3a**, **3d**, and **3i** of these two series have the best activating hTERT expression and compares favorably in relative SEAP activity and relative viability in Table 3 and Figure 4. The optimal substituents of the various homologues of 1,5-bisacyloxyanthraquinone were propionyloxy (**3a**), pivaloyoxy (**3d**), and 2,4-dichlorobenzoyl (**3i**), and 1,5-bisthio anthraquinones did not exhibit any significant activation hTERT expression. Anthraquinone derivatives are known for their antitumor activities. Here, we found that about half ($^{14}/_{30}$) of the compounds

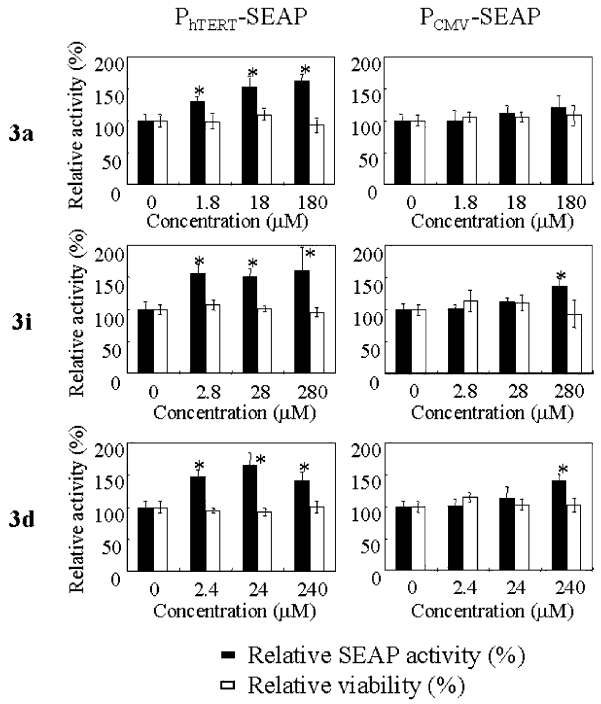


Figure 4. Specific activation of P_{hTERT}-SEAP by analogues of anthraquinone. About 2×10^3 cells of hTERT-BJ1 harboring P_{hTERT}-SEAP or P_{CMV}-SEAP were seeded in 96-well plates and incubated at 37 °C for 24 h. Cells were then washed with PBS, recultured in fresh media, and incubated with varying amounts of **3i**, **3a**, or **3d** for another 48 h. The culture media were collected and subjected to SEAP activity analysis. The level of cell growth was also determined using MTT assay. The values are obtained from six experiments using the values without drug treatment as 100%.

Table 3. Effects of Anthraquinones on the CMV Promoter Activity

		P_{CMV}-SEAP (hTERT-BJ1)[b]	
compd	concn[a] (μM)	relative viability (%)	relative SEAP activity (%)
2f	2.2	108 ± 9	103 ± 8
	22	109 ± 4	102 ± 10
	220	48 ± 6	122 ± 8
2j	2.0	116 ± 13	103 ± 15
	22	67 ± 18	102 ± 8
	220	52 ± 7	100 ± 8
2n	2.2	107 ± 10	112 ± 15
	22	118 ± 9	105 ± 16
	220	78 ± 18	120 ± 14
3a	2.8	114 ± 16	101 ± 6
	28	110 ± 12	112 ± 6
	280	94 ± 21	138 ± 12
3d	2.4	115 ± 7	102 ± 10
	24	104 ± 8	114 ± 18
	240	103 ± 11	141 ± 11
3i	1.8	105 ± 8	99 ± 15
	18	105 ± 7	111 ± 11
	180	108 ± 15	121 ± 17

[a] Values are in μM and represent an average of three experiments. The variance for the relative viability (%) and relative SEAP activity (%) values was less than ±20%. Activity of P_{CMV}-SEAP (hTERT-BJ1) cell growth was significantly different from that of the control; $n = 3$ or more, $P < 0.01$. Relative percentage of inhibition was not compared with that of the control, $P < 0.01$, mean ± SE, $n = 4$. Values are the mean percent activity at the indicated concentration and include standard errors. [b] CMV (cytomegalovirus); SEAP (secreted alkaline phosphatase).

preferentially inhibit the growth of a cancer cell line H1299 compared with the skin fibroblast BJ-1. The inhibitory effect does not seem to correlate with the modifying groups. Also, these effects are very sensitive to a slight modification of anthraquinone derivatives. Thus, it appears that the anthraquinone chromophore itself causes this activity. Finally, the effects of anthraquinones on the cytotoxicity, the telomerase-repression activity in cancer cells, and the telomerase-activation activity in normal cells are very diverse. A simple correlation between the modifications of anthraquinones with their biological effects cannot be achieved. The molecular mechanisms behind these diversified biological effects are unclear. In light of these findings, it is suggested that activation of hTERT expression by some of the anthraquinones is not sufficient for potent proliferative action. Nevertheless, the potential application of these compounds in tumor biology, stem cell research, and tissue engineering made them a group of compounds that are worth an extensive study.

Experimental Section

Melting points were determined with a Büchi 530 melting point apparatus and are uncorrected. All reactions were monitored by TLC, which were performed on precoated sheets on silica gel 60 F_{254}, and flash column chromatography was done in silica gel (E. Merck, 70−230 mesh) with CH_2Cl_2 as eluant, unless otherwise stated. ^{1}H NMR spectra were recorded with a Varian GEMINI-300 (300 MHz). δ values are in ppm relative to a tetramethylsilane internal standard. Fourier transform IR spectra (KBr) were recorded on a Perkin-Elmer 983G spectrometer. Mass spectra (EI, 70 eV, unless otherwise stated) were obtained on a Finnigan MAT TSQ-46 and Finnigan MAT TSQ-700.

The following anthraquinones were prepared by previously described procedures: **2** and **3**. All other compounds were commercial materials.

Cell Culture and Assessment of hTERT. Nonsmall lung cancer cells H1299 (telomerase positive) were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The hTERT immortalized hTERT-BJ1 (BD Biosciences Clontech)[39] were grown in DMEM media supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100 mg/mL streptomycin, 1 mM sodium pyruvate, and 4 mM L-arginine in a humidified atmosphere with 5% CO_2 at 37 °C. Culture media were changed every 3 days. To establish stable cell lines that the expression of hTERT could be monitored by a reporter system, a ~3.3 kbp DNA fragment ranging from −3338 to +1 bp of the hTERT gene was subcloned upstream to a secreted alkaline phosphatase gene (SEAP) and transfectectd into H1299 or hTERT-BJ1 by electroporation. The stable clones were selected using G418. The stable clones derived from H1299 or hTERT-BJ1 were cultured using conditions that are similar to their parental cells.

Cytotoxicity Assay. The tetrazolium reagent (MTT; 3-(4,5-di-methylthiazol)-2,5-diphenyltetrazolium bromide, USB) was designed to yield a colored formazan upon metabolic reduction by viable cells.[43,44] Approximately 2×10^3 cells were plated onto each well of a 96-well plate and incubated in 5% CO_2 at 37 °C for 24 h. To assess the in vitro cytotoxicity, each compound was dissolved in DMSO and prepared immediately before the experiments and was diluted into the complete medium before addition to cell cultures. Test compounds were then added to the culture medium for designated various concentrations. After 48 h, an amount of 25 μL of MTT was added to each well, and the samples were incubated at 37°C for 4 h. A 100 μL solution of lysis buffer containing 20% SDS and 50% *N*,*N*-dimethylformamide was added to each well and incubated at 37 °C for another 16 h. The absorbency at 550 nm was measured using an ELISA reader.

Telomerase Assay. Telomeric repeat amplification protocol (TRAP) was utilized for telomerase activity assay.[45−47] The telomerase products were resolved by 10% polyacrylamide gel electrophoresis and visualized by staining with SYBER Green. As a source of telomerase, the total cell lysates derived from lung cancer cell line H1299 cells were used. Protein concentration of the lysates was assayed using Bio-Rad protein assay kit using BSA standards.

SEAP Assay.[48] Secreted alkaine phosphatase was used as the reporter system to monitor the transcriptional activity of hTERT. Here, about 10^4 cells each were grown in 96-well plates and incubated at 37 °C for 24 h and changed with fresh media. Varying amounts of drugs were added, and cells were incubated for another 24 h. Culture media were collected and heated at 65 °C for 10 min to inactivate heat-labile phosphatases. An equal amount of SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, and 20 mM L-homoarginine) was added to the media, and *p*-nitrophenyl phosphate was added to a final concentration of 12 mM. Absorptions at 405 nm were taken, and the rate of absorption increase is determined.

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