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Human telomerase inhibition and cytotoxicity of regioisomeric disubstituted amidoanthraquinones and aminoanthraquinones

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Abstract—Telomerase is an attractive target for the rational design of new anticancer drugs due to its central role in the control of cellular proliferation. A number of 1,4-disubstituted amidoanthraquinones and 1,5-disubstituted aminoanthraquinones that are related to mitoxantrone and ametantrone have previously been prepared. The present study details the effects on human telomerase of these new classes of 1,4- and 1,5-difunctionalized tricyclic anthraquinone compounds. We have used cytotoxicity assay, reporter SEAP assay to monitor the hTERT expression, and TRAP-G4 assay to measure the relative activity of these compounds, and have examined how the attached substituents affect their ability to influence telomerase. Cytotoxicity levels in human tumor cell lines were at comparable levels for several compounds. Structural and activity relationships indicated that the position of disubstituent side chains is important for its inhibitory effect. Moreover, a primary amine or tertiary amine on the substitution group appears to be required for the telomerase inhibitory effect. There is no significant correlation between telomerase activity and cytotoxicity. These symmetrical disubstituted anthraquinones may represent useful leads for the development of human telomerase inhibitors as potential anticancer agents, and the exact mode of intercalative binding is dictated by the positional placement of substituent side chains for effective telomerase inhibition.

1. Introduction

Telomerase inhibitors have been touted as a novel cancer specific therapy, as most tumor cells have high expression of telomerase, whereas most normal somatic cells express low or undetectable levels of telomerase.[1] Telomeres, the ends of eukaryotic chromosomes, are characterized by simple G-rich repeating sequences exemplified by the human telomeric sequence 5′-TTAGGG.[2,3] Telomerase is a specialized reverse transcriptase responsible for the maintenance of telomere, in which a short sequence acts as the template for synthesis of telomeric DNA.[2] The tandem repeating guanine-rich nucleic acid sequences serve to protect chromosomal stability and maintain integrity.[4] Telomerase activity has been detected in 85–90% of all human cancers and may be essential for cell immortality.[5,6] Most immortalized cell lines and malignant human tumors appear to maintain constant telomere length via telomerase activity.[7] In contrast, normal human cells that lack telomerase activity progressively lose telomere sequences. Progressive telomere shortening eventually triggers an irreversible arrest of proliferation called cellular senescence. These observations have led to a model in which telomere length reflects the mitotic history of somatic cells.[8] The catalytic protein subunit of telomerase, hTERT, has been identified,[9,10] and it appears that hTERT expression is the key regulator for telomerase activity.[11] Introduction of the hTERT gene into several normal human cells leads to immortalization of these cells.[12–15] In human, telomerase activity is tightly regulated by expression of the human telomerase reverse transcriptase (hTERT) gene.[16] It appears to be stringently repressed in normal human somatic tissues but reactivated in cancer and immortal cells.[6] Because telomerase is required for the sustained proliferation of most immortal cells, including cancer

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cells, it has become the focus of much attention as a novel and potentially highly-specific target for the development of new anticancer chemotherapeutics. Telomerase plays a key role in the maintenance of chromosomal stability in tumours, but it still remains unknown whether anticancer agents can inhibit telomerase activity.[17]

There is significant interest in the development of inhibitors of human telomerase for the treatment of cancer.[4,18–22] For example, 1,4-bis-piperidino disubstituted amidoanthraquinones have been shown to inhibit telomerase, probably by stabilizing guanine-quadruplex complexes formed by telomeric DNA sequences.[23] Telomerase activation in some cell types appears to offset proliferation-dependent telomere shortening, delaying the inherent mitotic clock.[24] Several studies have already investigated adrenocortical cells,[25] endothelial cells,[26] and bone marrow stromal stem cells in vitro by introducing hTERT into these cells and restoring the cellular function.[27,28] Thus, inhibition or activation of the reverse transcriptase telomerase could profoundly affect the proliferative capacity of normal cells and cancers.[29] Anthraquinone-base compounds are important chromophore in cancer chemotherapy. An area of particular activity and promise is concerned with aminoalkyl- and amidosubstituted anthraquinones[30–34] with the demonstration of clinical activity for the 1,4-disubstituted compounds mitoxantrone and ametantrone (Chart 1).[35–37] The mechanism of action of mitoxantrone and other aminoalkyl anthraquinone involves multiple effects on cellular DNA, so strong DNA binding through intercalation is believed to be responsible for the pharmacological activity of these agents.[38,39] The cytotoxicity of anthraquinone mitoxantrone is thought to relate to its trapping of DNA topoisomerase II complexes on cellular DNA.[40] Additionally, a number of studies have indicated that anthraquinone chromophore structure may also play a major role in lipid peroxidation.[41,42]

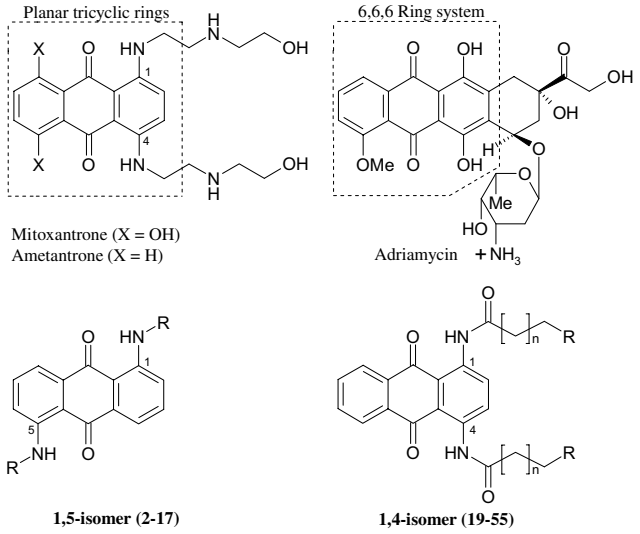

Chart 1.

Our previous studies have shown that several of these symmetrically disubstituted anthraquinone analogs activation of human telomerase can reverse transcriptase expression.[11] These results provide a new clue on the effects of the anthraquinone compounds on the modulation of hTERT gene expression. The present study explores the effects on hTERT expression using cell-based assay systems with distinct series of regioisomeric difunctionalized aminoanthraquinones and amidoanthraquinones substituted at the 1,4-, and 1,5-positions (Chart 1). We identify several compounds that repressed hTERT expression in cancer cells and activate hTERT expression in normal cells, although these compounds also affect the expression of gene under the control of a CMV promoter. We conclude that these compounds with anthraquinone moiety could have a role in both activation and repression of hTERT expression. We also describe the inhibitory activity of telomerase with these compounds. This study enables us to address the issue of how, and to what extent, the position of side chain substituents affects telomerase activity. Structure–activity relationship studies show the crucial role of diamino- and diamido-substituted groups in side chains of anthraquinones for the telomerase modulation. However, the importance of this group for the action mechanisms of anthraquinones is still not clear.

2. Results and discussion

The marked antineoplastic activity displayed by a number of bis(substituted aminoalkylamino)anthraquinones,[31] particularly mitoxantrone, ametantrone and adriamycin promoted a structural modification study in our laboratory. On the basis of previously established structure–activity relationships (SARs) for compounds that are able to inhibit topoisomerase II or interact with G-quadruplex to varying degrees, we have now designed and synthesized anthraquinones with symmetrical bisamino- and bisamido substituted side chains, and several additional anthraquinones with both side chains replaced by a similar substitutions. In addition, several biologically interesting ring systems or its analogs were also prepared for this investigation.

Telomerase enzyme activity is essential for the sustained proliferation of most immortal cells, including cancer cells, and is currently an important recognized target for the development of novel and potentially tumor-specific anticancer chemotherapeutics.[4] Recent advances in telomerase inhibition have been achieved by using antisense oligonucleotides and ribozymes to target the telomerase mRNA or the telomerase RNA template. Also, small molecules are potent catalytic inhibitors of telomerase.[43] Substitutions on the planar ring of anthraquinone have been shown to have biological effects.[30,44–50] Previously we showed that 9-acyloxy 1,5-dichloroanthracene derivatives and 1,5-bisacyloxyanthraquinones did not have any specific telomerase inhibitory activity.[11,51] Compounds with side chains -$(CH_2)_2NH(CH_2)_2$-OH **6** and -$(CH_2)_3NH_2$ **10**, show inhibition of G-quadruplex-induced telomerase at the IC_{50} levels of 0.2 and 0.5 μM, respectively. These molecules are the most

potent telomerase inhibitors in this series of compounds. In contrast to the structurally related molecules 1,5-disubstituted anthraquinones that were recently shown to activate telomerase expression in normal cells, only compound **42** selectively activated telomerase expression in normal cells. Interestingly, some of these compounds of 1,5-bisacyloxy side arms activated hTERT expression in normal cells, raising the possibility that these compounds might also have a role in cell immortalization.[11] Thus, we focus our attention on the role of our newly synthesized 1,4-diamidoanthraquinones and 1,5-diaminoanthraquinones on hTERT expression. These compounds resemble the anticancer agents mitoxantrone and ametantrone (Chart 1), and were prepared by the condensation of substituted 1,5-dichloroanthraquinone with appropriate amines, and acylation of 1,4-diaminoanthraquinone with appropriate acyl chlorides, some followed by amination. Since the expression of human telomerase catalytic component hTERT is the key regulator in telomerase activity,[52] we analyzed the expression of telomerase by monitoring the expression of hTERT as the criteria.

The analysis systems used cell lines derived from H1299 (non-small cell lung cancer cells) and hTERT-BJ1 (human normal skin fibroblast cells immortalized with hTERT gene) cells. These cells were generated by introducing H1299 or hTERT-BJ1 cells with DNA constructs harboring P_{hTERT}-SEAP, so that the 3.4 kbp hTERT promoter fused upstream to a reporter gene, SEAP.[11] We have shown that the 3.4 kbp DNA fragment used in our study is sufficient to confer the properties of hTERT promoter.[11]

The expression of SEAP in H1299 cells harboring P_{hTERT}-SEAP was used as the criteria 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 and by analysis of the symmetrical 1,5-diaminoanthraquinones on hTERT expression in these cells. As shown in Table 1, most of the compounds did not show significant differences from mitoxanthrone on cell viability. Only compound **10** showed >10 fold more sensitive cytotoxicity than that of mitoxantrone in both cells. Under the situation that these compounds did not affect cell viability, we found that two compounds, **12** and **17**, activated the SEAP expression in hTERT-BJ1 cells (Table 1). It is also interesting to note that even though compounds **5** and **12** appeared to be relatively toxic to H1299 cells, they slightly repressed hTERT expression in these cells. To test if these three compounds affect the expression of other promoters, we analyzed the effects of these three compounds on the activity of a CMV promoter. The CMV promoter-driven SEAP (P_{CMV}-SEAP), was introduced into hTERT-BJ1 or H1299, to generate stable cell lines and used these cell lines for analysis.[11] All of these compounds affected the expression of CMV promoter, suggesting that the effects of these three compounds on hTERT promoter are not specific (data not shown).

We next evaluate the biological effects of 1,4-diamidoanthraquinones in our systems. As shown in Table 2, most of the compounds did not show significant differences from mitoxantrone on cell viabilities. Under the condition that these compounds did not affect cell viability, two compounds, **19** and **42**, slightly activated SEAP activity in both cells. Significantly, compound **48** was capable of repressing the SEAP expression in H1299 cells (Table 2). Thus, our results indicate that 1,4-diamidoanthraquinones were capable of affecting hTERT expression by activation or repression. While compounds **19** and **48** appear to affect hTERT promoter nonspecifically, the effect of compound **42** on hTERT promoter is specific since it does not affect the expression of SEAP under the control of a CMV promoter (data not shown).

G-quadruplex-binding agents have been shown to inhibit telomerase activity through the stabilization of the G-quadruplex structure.[43] These structures are proposed to exist in vivo, although direct confirmatory evidence is lacking. Guanine-rich regions of DNA capable of forming G-quadruplex structures are found in a variety of chromosomal regions, including telomeres and promoter regions of DNA.[53] DNA is the molecular target for many of the drugs that are used in cancer therapeutics, and is viewed as a non-specific target of cytotoxic agents. Furthermore, a new generation of agents that target DNA-associated processes are anticipated to be far more specific and effective.[54] There are several anthraquinones which have been reported to bind to G-quadruplex DNA formed by telomeric DNA and to inhibit telomerase activity.[19,21,34,55,56] In addition, a modified telomerase assay (TRAP-G4) has been used to evaluate the effects of anthraquinones on G-quadruplex-nduced telomerase activity.[57] In the TRAP-G4 assay, a G-quadruplex sequence was introduced into the telomerase extension primer that is susceptible to forming an intramolecular G-quadruplex. Because the formation of G-quadruplex blocks telomerase extension, this TRAP-T4 assay enables the evaluating the effects of G-quadruplex stabilizing agents on telomerase activity. Among all the anthraquinones evaluated, we found that compounds **5**, **6**, **9**, **10**, **12**, **23**, and **48** showed telomerase inhibitory effect at the concentrations of 2.0 and 40 μM, respectively. More significantly, compounds **6** and **10** showed an IC_{50} of 0.2 and 0.5 μM, making them the most potent telomerase inhibitors in this series of compounds.[11,21,22,34,55,56] It is also interesting to note that mitoxantrone also inhibits telomerase very effectively (Fig. 1). However, the finding that mitoxantrone retains significant toxicity towards leukaemia cells may suggest that DNA is perhaps not the unique molecular target.[58]

3. Conclusions

Since telomerase is expressed in essentially all cancer cells, but not in most normal human cells, specific telomerase inhibition has potential as a universal anticancer therapy with few side effects.[59] Regulation of telomerase activity could be an important mechanism for limiting the growth of normal and cancer cells.[60] Inhibition of telomerase activity has long been proposed as a means to

Table 1. Dual Effects of Symmetrical 1,5-Diaminoanthraquinones on Activating or Repressing hTERT Expression

Compd	R	Concn. (μM)[a]	P_{hTERT}-SEAP (hTERT-BJ1)[b]		P_{hTERT}-SEAP (H1299)[c]		TRAP assay IC_{50} (μM)
			Viability (IC_{50})	Relative SEAP activity (%)	Viability (IC_{50})	Relative SEAP activity (%)	
2	CH_2CH_3	3.3	100 ± 4.8	94 ± 17.4	106 ± 7.4	108 ± 6.3	
		33	97 ± 1.0	96 ± 8.5	104 ± 3.7	101 ± 6.8	>339
		339	99 ± 3.2	66 ± 11.8	103 ± 6.4	95 ± 11.0	
3	CH_2CH_2OH	3.0	116 ± 5.6	45 ± 20.6	105 ± 6.3	91 ± 3.9	
		30	90 ± 26.9	16 ± 13.1	88 ± 9.1	88 ± 4.5	>308
		308	69 ± 18.7	11 ± 11.6	53 ± 2.8	5.7 ± 1.1	
4	$CH(CH_3)_2$	3.1	101 ± 4.5	32 ± 18.3	114 ± 7.6	94 ± 4.1	
		31	93 ± 6.5	23 ± 20.5	111 ± 4.7	93 ± 2.0	>310
		310	98 ± 12.9	(−2) ± 15.3	50 ± 7.5	66 ± 4.7	
5	$CH_2CH_2N(CH_3)_2$	2.6	92 ± 4.8	11 ± 22.4	107 ± 9.1	103 ± 6.0	
		26	76 ± 5.9	(−15) ± 18.2	81 ± 8.3	54 ± 5.9	20
		262	7 ± 18.2	(−26) ± 16.9	29 ± 2.6	40 ± 5.9	
6	$CH_2CH_2NH(CH_2)_2OH$	2.4	84 ± 19.8	60 ± 11.6	97 ± 8.7	87 ± 3.8	
		24	60 ± 11.2	40 ± 17.3	37 ± 3.9	43 ± 5.5	0.2
		242	44 ± 12.9	52 ± 19.1	11 ± 4.1	40 ± 6.7	
7	$CH_2CH_2CH_3$	3.1	97 ± 5.6	18 ± 16.7	99 ± 7.6	101 ± 15.3	
		31	93 ± 8.9	19 ± 24.6	98 ± 5.8	100 ± 5.6	>310
		310	42 ± 8.1	(−7) ± 27.1	46 ± 9.5	89 ± 8.3	
8	$CH_2CH(CH_3)_2$	2.8	110 ± 7.6	22 ± 18.3	108 ± 4.8	94 ± 7.0	
		28	103 ± 3.0	22 ± 21.4	106 ± 3.7	91 ± 2.5	>285
		285	72 ± 3.3	29 ± 3.9	80 ± 4.8	75 ± 4.1	
9	$CH_2CH_2CH_2OH$	2.8	72 ± 7.4	41 ± 12.5	107 ± 5.0	88 ± 4.9	
		28	39 ± 10.5	0 ± 22.1	49 ± 3.5	69 ± 5.4	30
		282	26 ± 15.9	(−3) ± 10.0	40 ± 10.4	33 ± 2.2	
10	$CH_2CH_2CH_2NH_2$	2.8	85 ± 6.9	103 ± 19.8	85 ± 11.5	102 ± 4.9	
		28	3 ± 6.9	47 ± 20.5	32 ± 9.3	44 ± 7.5	0.5
		283	(−2) ± 7.7	60 ± 15.1	16 ± 2.1	38 ± 6.0	
11	$CH_2CH_2CH_2CH_3$	2.8	109 ± 9.1	98 ± 27.4	102 ± 7.0	106 ± 7.1	
		28	99 ± 5.9	103 ± 27.0	104 ± 9.2	105 ± 5.5	>285
		285	75 ± 5.9	123 ± 22.9	81 ± 13.9	98 ± 5.2	
12	$CH_2CH_2CH_2CH_2NH_2$	2.6	101 ± 10.5	114 ± 20.5	114 ± 8.1	89 ± 4.9	
		26	101 ± 8.8	113 ± 21.6	110 ± 7.1	71 ± 9.8	5
		265	91 ± 11.8	127 ± 19.9	16 ± 3.4	27 ± 2.0	
13	$CH_2CH_2CH_2CH_2CH_2CH_3$	2.4	106 ± 3.9	90 ± 19.5	99 ± 5.3	101 ± 6.1	
		24	97 ± 4.6	97 ± 17.9	95 ± 6.2	106 ± 9.1	120
		245	68 ± 8.5	122 ± 30.2	85 ± 8.8	104 ± 9.4	
14	cyclopentane	2.6	104 ± 3.6	18 ± 20.1	114 ± 7.5	98 ± 4.5	
		26	102 ± 4.0	14 ± 29.6	110 ± 5.7	95 ± 2.8	200
		267	87 ± 4.6	34 ± 16.5	86 ± 8.2	90 ± 4.8	
15	2,3-$(CH_3)_2$-cyclohexane	2.1	118 ± 10.6	94 ± 20.2	88 ± 7.9	103 ± 9.2	
		21	99 ± 8.2	92 ± 17.4	84 ± 10.2	105 ± 5.1	200
		218	84 ± 6.9	90 ± 22.7	58 ± 8.0	96 ± 4.5	
16	$CH_2C_6H_5$	2.3	110 ± 4.1	110 ± 14.8	98 ± 8.4	99 ± 5.0	
		23	102 ± 5.3	92 ± 13.8	100 ± 7.3	103 ± 8.9	>238
		238	74 ± 4.9	86 ± 13.8	40 ± 8.4	97 ± 3.8	
17	$CH_2CH_2C_6H_5$	2.2	97 ± 4.0	115 ± 12.9	94 ± 5.2	105 ± 3.0	
		22	92 ± 1.0	103 ± 12.3	97 ± 3.2	103 ± 9.5	>223
		223	81 ± 4.7	133 ± 23.8	77 ± 4.2	96 ± 2.9	
	Mitoxantrone	1.9	75 ± 2.9	30 ± 5.8	100 ± 5.6	81 ± 3.8	
		19	56 ± 3.1	13 ± 9.2	57 ± 4.3	66 ± 4.0	2
		193	10 ± 2.0	4 ± 14.2	39 ± 3.2	47 ± 3.9	

[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 (hTERT-BJ1) cell growth was significantly different from that of the control; $n = 3$ or more, $P < 0.05$.

[b] The hTERT immortalized hTERT-BJ1 was purchased from BD Biosciences Clontech. Note: The results in this column are shown as means ± SE of experiments repeated five times. The different symbols qualify as in any concentration of treatment: relative cell viability >80%, relative SEAP activity >100% and *P* value below 0.05 analyzed with Two-tail T-test. The ratio of relative cell viability under relative SEAP activity is over 1.2. All of SEAP data are shown as the result that drug-self interference has been subtracted.

[c] Repression of P_{hTERT}-SEAP (hTERT-H1299) cell growth was significantly different from that of the control; $n = 3$ or more, $P < 0.05$. Relative percentage of inhibition was not compared with that of the control, $P < 0.01$, mean ± S.E., $n = 4$. Values are mean percent activity at the indicated concentration, and standard errors.

Table 2. Dual Effects of Symmetrical 1,4-Diamidoanthraquinones on Activating or Repressing hTERT Expression

Compd	R	Concn. (μM)[a]	P_{hTERT}-SEAP (hTERT-BJ1)[b]		P_{hTERT}-SEAP (H1299)[c]		TRAP assay IC_{50} (μM)
			Relative MTT Viability (%)	Relative SEAP activity (%)	Relative MTT viability (%)	Relative SEAP activity (%)	
19	CH_2Cl	2.5	101 ± 2.7	123 ± 16.8	104 ± 5.5	102 ± 5.3	
		25	84 ± 6.3	129 ± 17.7	96 ± 3.8	110 ± 8.8	>255
		255	44 ± 4.7	121 ± 10.9	21 ± 1.5	97 ± 7.1	
20	$(CH_2)_2Cl$	2.3	102 ± 4.1	36 ± 12.1	99 ± 6.0	87 ± 2.3	
		23	82 ± 8.2	40 ± 10.8	68 ± 2.4	87 ± 3.1	>238
		238	60 ± 2.5	14 ± 11.8	30 ± 3.8	68 ± 5.3	
21	$CH(Cl)CH_3$	2.3	94 ± 10.4	91 ± 10.8	111 ± 5.5	106 ± 5.5	
		23	68 ± 8.2	101 ± 8.4	77 ± 3.4	109 ± 1.0	>238
		238	48 ± 2.3	81 ± 8.5	11 ± 1.4	85 ± 5.3	
22	$CH_2N(CH_2CH_3)_2$	2.1	105 ± 2.8	111 ± 24.4	105 ± 4.9	98 ± 3.3	
		21	86 ± 3.5	120 ± 25.6	87 ± 3.9	86 ± 7.4	200
		215	32 ± 13.1	83 ± 19.0	44 ± 2.7	48 ± 2.3	
23	$CH_2CH_2N(CH_2CH_3)_2$	2.0	83 ± 9.2	2 ± 11.1	92 ± 6.1	73 ± 2.7	
		20	15 ± 3.9	(−2) ± 11.2	11 ± 2.5	53 ± 2.7	2
		203	6 ± 3.9	(−2) ± 6.5	1 ± 2.4	39 ± 0.8	
24	$CH(CH_3)N(CH_2CH_2)_2$	2.0	97 ± 3.9	40 ± 14.5	96 ± 7.9	96 ± 14.3	
		20	52 ± 7.2	49 ± 9.3	77 ± 7.2	104 ± 6.0	>203
		203	36 ± 5.8	22 ± 6.6	75 ± 4.6	73 ± 11.7	
25	$CH(CH_3)NHCH_2C_3H_5$	2.0	103 ± 6.4	91 ± 19.4	106 ± 4.1	123 ± 12.4	
		20	92 ± 11.4	95 ± 14.2	64 ± 5.1	112 ± 11.5	>203
		204	104 ± 17.4	65 ± 15.7	30 ± 4.7	80 ± 14.5	
26	$CH_2CH_2NHCH_2C_3H_5$	2.0	95 ± 2.5	97 ± 13.8	108 ± 7.8	95 ± 4.2	
		20	84 ± 1.5	124 ± 22.8	83 ± 1.6	91 ± 8.9	ND
		204	35 ± 3.7	105 ± 17.7	67 ± 9.8	52 ± 4.4	
27	CH_3	3.1	117 ± 3.0	77 ± 12.4	109 ± 3.5	85 ± 2.3	
		31	95 ± 11.9	83 ± 10.0	108 ± 3.6	90 ± 3.3	>310
		310	49 ± 8.9	59 ± 15.2	37 ± 1.3	80 ± 3.1	
28	cyclopropane	2.6	103 ± 6.8	64 ± 9.5	95 ± 4.2	108 ± 4.9	
		26	82 ± 5.9	54 ± 8.8	88 ± 4.2	106 ± 7.3	>267
		267	58 ± 4.1	43 ± 8.3	41 ± 4.6	92 ± 5.9	
29	cyclopentane	2.3	106 ± 2.3	85 ± 7.7	107 ± 6.1	119 ± 8.5	
		23	103 ± 4.4	93 ± 18.5	103 ± 6.1	118 ± 9.2	>233
		233	93 ± 5.7	63 ± 36.5	92 ± 8.6	113 ± 8.9	
30	cyclohexane	2.1	102 ± 3.0	65 ± 16.0	104 ± 6.4	104 ± 1.8	
		21	97 ± 2.4	42 ± 24.3	96 ± 5.8	100 ± 6.2	>218
		218	83 ± 4.2	56 ± 16.3	67 ± 1.5	93 ± 2.5	
31	$(CH_2)_2C_5H_9$	2.0	107 ± 3.6	90 ± 9.2	105 ± 2.1	111 ± 9.4	
		20	101 ± 4.7	87 ± 5.5	106 ± 4.7	116 ± 8.0	>205
		205	100 ± 5.8	87 ± 8.0	95 ± 1.3	109 ± 9.2	
32	$2\text{-}SC(CH)_3$	2.1	118 ± 10.6	94 ± 20.2	108 ± 3.7	100 ± 3.1	
		21	99 ± 8.2	92 ± 17.4	107 ± 8.5	105 ± 5.9	>218
		218	84 ± 6.9	90 ± 22.7	84 ± 4.3	97 ± 4.9	
33	$2\text{-}OC(CH)_3$	2.3	91 ± 9.5	39 ± 7.8	101 ± 6.0	99 ± 7.8	
		23	69 ± 10.7	45 ± 11.0	75 ± 3.9	97 ± 4.6	>234
		234	72 ± 11.3	37 ± 15.0	32 ± 5.1	90 ± 2.9	
34	$CH_2\text{-}2\text{-}SC(CH)_3$	2.0	116 ± 7.5	53 ± 17.4	104 ± 8.0	111 ± 3.7	
		20	105 ± 4.4	40 ± 12.7	83 ± 6.6	115 ± 4.3	100
		205	76 ± 6.5	14 ± 24.1	41 ± 4.0	102 ± 5.5	
35	C_6H_5	2.2	95 ± 4.9	−4 ± 25.7	109 ± 6.0	81 ± 3.3	
		22	69 ± 3.2	−10 ± 26.8	88 ± 6.2	84 ± 2.6	>224
		224	38 ± 2.6	−41 ± 26.8	27 ± 1.8	80 ± 2.7	
36	$3\text{-}CH_3C_6H_4$	2.1	110 ± 2.6	97 ± 17.1	101 ± 5.6	107 ± 4.6	
		21	109 ± 4.6	78 ± 7.1	97 ± 6.2	104 ± 4.0	>210
		210	101 ± 6.0	70 ± 5.8	83 ± 7.8	99 ± 9.1	
37	$2\text{-}FC_6H_4$	2.0	106 ± 6.5	95 ± 8.9	116 ± 2.4	105 ± 7.8	
		20	105 ± 8.5	96 ± 9.8	104 ± 6.1	107 ± 11.9	>207
		207	86 ± 7.3	79 ± 7.4	77 ± 2.6	103 ± 14.4	
38	$3\text{-}FC_6H_4$	2.0	102 ± 1.5	107 ± 11.9	105 ± 6.8	102 ± 2.9	
		20	97 ± 2.9	108 ± 15.5	96 ± 7.4	110 ± 6.5	>207
		207	85 ± 2.6	95 ± 13.9	66 ± 5.5	106 ± 9.4	
39	$4\text{-}FC_6H_4$	2.0	103 ± 9.0	104 ± 16.3	110 ± 5.9	111 ± 3.6	
		20	107 ± 3.1	101 ± 27.6	103 ± 6.6	107 ± 4.8	>207
		207	83 ± 5.3	100 ± 14.5	89 ± 6.7	107 ± 9.0	
40	$2\text{-}ClC_6H_4$	1.9	116 ± 7.7	110 ± 20.1	114 ± 5.4	108 ± 10.5	

(*continued on next page*)

Table 2 (*continued*)

Compd	R	Concn (μM)[a]	P_{hTERT}-SEAP (hTERT-BJ1)[b]		P_{hTERT}-SEAP (H1299)[c]		TRAP assay IC_{50} (μM)
			Relative MTT Viability (%)	Relative SEAP activity (%)	Relative MTT viability (%)	Relative SEAP activity (%)	
		19	109 ± 2.2	96 ± 33.4	72 ± 3.6	105 ± 7.7	>194
		194	95 ± 2.0	95 ± 36.6	60 ± 7.9	100 ± 8.9	
41	3-ClC_6H_4	1.9	99 ± 9.8	98 ± 10.1	95 ± 11.3	110 ± 4.9	
		19	90 ± 1.9	105 ± 8.8	102 ± 8.5	112 ± 6.1	>194
		194	60 ± 2.0	89 ± 10.1	59 ± 4.3	91 ± 3.0	
42	4-ClC_6H_4	1.9	111 ± 0.8	116 ± 28.6	89 ± 5.5	109 ± 6.8	
		19	103 ± 5.9	112 ± 21.9	89 ± 9.5	118 ± 5.6	>194
		194	99 ± 5.2	152 ± 39.7	79 ± 5.6	116 ± 12.3	
43	2-$NO_2C_6H_4$	1.8	110 ± 4.1	102 ± 33.4	104 ± 6.3	105 ± 4.3	
		18	107 ± 6.3	122 ± 19.5	101 ± 4.1	107 ± 7.9	>186
		186	99 ± 3.2	114 ± 28.1	86 ± 5.3	94 ± 8.0	
44	4-$CF_3C_6H_4$	1.7	98 ± 4.4	100 ± 15.1	98 ± 7.8	110 ± 5.5	
		17	100 ± 3.4	90 ± 16.3	100 ± 8.9	114 ± 5.6	>171
		171	89 ± 3.6	103 ± 17.6	77 ± 6.7	96 ± 2.1	
45	2,5-$(CF_3)_2C_6H_3$	1.3	107 ± 6.4	16 ± 31.8	99 ± 8.6	106 ± 6.7	
		13	85 ± 4.8	24 ± 21.5	79 ± 10.3	105 ± 7.9	>139
		139	56 ± 4.6	26 ± 36.9	37 ± 8.8	94 ± 10.2	
46	2,4-$F_2C_6H_3$	1.9	104 ± 5.6	48 ± 21.7	87 ± 3.7	93 ± 5.6	
		19	101 ± 4.4	51 ± 18.5	85 ± 7.1	99 ± 5.4	>192
		192	103 ± 6.1	48 ± 23.1	69 ± 8.4	99 ± 1.8	
47	2,4-$Cl_2C_6H_3$	1.7	102 ± 3.4	33 ± 11.2	106 ± 3.9	85 ± 7.9	
		17	98 ± 7.2	25 ± 17.2	95 ± 1.3	84 ± 7.1	>171
		171	76 ± 4.8	39 ± 12.8	83 ± 1.2	87 ± 2.6	
48	2,4,6-$Cl_3C_6H_2$	1.5	98 ± 8.5	31 ± 23.0	113 ± 4.8	98 ± 4.5	
		15	63 ± 7.0	12 ± 12.5	107 ± 5.4	66 ± 2.4	40
		153	40 ± 22.2	4 ± 15	20 ± 3.3	41 ± 3.9	
49	2,3,6-$F_3C_6H_2$	1.8	102 ± 3.3	35 ± 14.1	111 ± 2.9	91 ± 5.2	
		18	90 ± 6.0	22 ± 24.4	89 ± 3.7	97 ± 4.1	>180
		180	70 ± 6.8	31 ± 7.9	55 ± 3.3	91 ± 3.3	
50	2,4,5-$F_3C_6H_2$	1.6	104 ± 4.5	117 ± 14.2	90 ± 6.6	109 ± 10.8	
		16	89 ± 5.0	114 ± 20.4	81 ± 1.9	118 ± 10.3	>161
		161	80 ± 3.8	115 ± 30.8	60 ± 5.0	104 ± 8.2	
51	2,3-Cl_2-5-FC_6H_2	1.6	111 ± 6.8	113 ± 16.0	98 ± 7.0	102 ± 4.1	
		16	110 ± 7.1	131 ± 19.1	103 ± 8.7	102 ± 5.1	>161
		161	101 ± 6.6	106 ± 17.3	87 ± 5.1	83 ± 4.0	
52	$CH(CH_2)CHC_6H_5$	1.8	92 ± 16.9	92 ± 16.9	101 ± 2.4	95 ± 7.7	
		18	97 ± 18.2	97 ± 18.2	100 ± 4.7	93 ± 3.2	>189
		189	104 ± 10.7	102 ± 16.6	94 ± 3.5.	87 ± 3.6	
53	$CH_2SC_6H_5$	1.8	115 ± 4.8	14 ± 14.7	104 ± 5.6	93 ± 3.2	
		18	105 ± 6.7	23 ± 9.5	96 ± 4.3	92 ± 3.5	>185
		185	60 ± 2.3	7 ± 9.6	59 ± 3.8	80 ± 4.0	
54	$CH_2C_6H_4F(p)$	1.9	107 ± 7.2	90 ± 18.4	108 ± 8.8	109 ± 10.8	
		19	100 ± 6.3	102 ± 20.0	107 ± 7.5	109 ± 5.8	>195
		195	96 ± 4.1	97 ± 11.6	78 ± 8.0	109 ± 6.6	
55	2,5-Dimethylfuran	2.0	101 ± 6.2	84 ± 13.3	87 ± 4.9	104 ± 10.8	
		20	101 ± 5.8	79 ± 14.7	71 ± 2.3	103 ± 10.0	>195
		207	93 ± 7.1	81 ± 16.1	47 ± 2.6	98 ± 10.5	
	Mitoxantrone	1.9	75 ± 2.9	30 ± 5.8	100 ± 5.6	81 ± 3.8	
		19	56 ± 3.1	13 ± 9.2	57 ± 4.3	66 ± 4.0	2
		193	10 ± 2.0	4 ± 14.2	39 ± 3.2	47 ± 3.9	

[a] Values are in μM nd 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 (hTERT-BJ1) cell growth was significantly different from that of the control; $n = 3$ or more, $P < 0.05$.
[b] The hTERT immortalized hTERT-BJ1 was purchased from BD Biosciences Clontech.
[c] Repression of P_{hTERT}-SEAP in H1299 cell growth was significantly different from that of the control; $n = 3$ or more, $P < 0.05$. Relative percentage of inhibition was not compared with that of the control, $P < 0.01$, mean ± S.E., $n = 4$. Values are mean percent activity at the indicated concentration, and standard errors.

selective targeting of cancer cells, whereas its activation has been proposed for *ex vivo* expansion of stem cells in tissue engineering. Our finding of anthraquinones in telomerase inhibition, hTERT activation or repression, and cytotoxic activities would indicte that these compounds may represent useful leads for the development of potentially highly selective target of human telomerase and a novel class of antiproliferative agents.

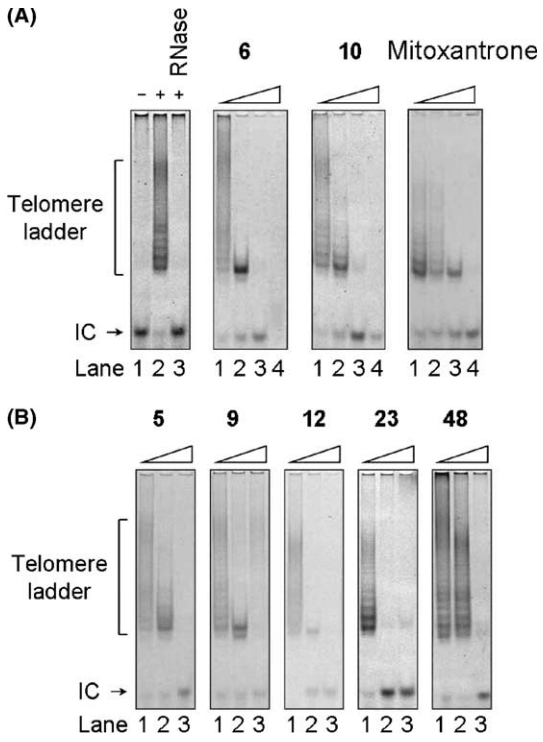

Figure 1. Inhibition of telomerase activity by some 1,5-diaminoanthraquinones and 1,4-diamidoanthraquinones. (A) TRAP-G4 assay was conducted using cell extracts prepared from H1299 cells and 2 μg of extracts were used in each assay. Extended products were separated on a 10% polyacrylamide gel and visualized with SYBER Green staining. The photo pictures of the results are presented. The concentration of test compounds were 0.02, 0.2, 2, 20 μM, respectively. (B) The experiments were conducted similar to that in (A) with the exception that the concentration of inhibitors were 2, 20, 200 μM, respectively for each compounds. Because the internal control (IC) shares one oligonucleotide with the reaction, the IC products became apparent when telomerase activities were inhibited.

As in the case of the anthracyclines, the mechanisms of cell killing for the anthraquinones are poorly understood. Interactions with DNA,[61,62] free-radical formation,[63] topoisomerase interactions,[64–66] and/or membrane interactions[67] may play roles in the expressed cytotoxicity.[50] Anthraquinone derivatives are potent antineoplastic chemotherapeutics. One part of this study was to elucidate the structure–activity relationships of these types of compounds. Here we found that about half (7/16) of the 1,5-diaminoanthraquinones and similar fractions (21/37) of the 1,4-diamidoanthraquinones preferentially inhibit the growth of the cancer cell line H1299, compared with the skin fibroblast BJ-1. Thus, it appears that 1,4- or 1,5-bis-substitution of anthraquinone might not determine the inhibitory effects on cancer cells. It is also noteworthy that compound **25** has an IC_{50} toward H1299 cells similar to that of mitoxantrone; however, it does not appear to cause growth inhibitory effect toward BJ-1 cells. This selective growth inhibitory property of compound **25** should make it a better drug for treating cancers. A structure–activity relationship study was also conducted on a number of aminoanthraquinones and amidoanthraquinones. This showed that both the position and the nature of the center nitrogen atom of the side chain are vital to the antineoplastic activity or telomerase activity. The 1,5-disubstituted aminoanthraquinones should be worthy of extensive analysis for future drug developments since several derivatives had very high antitumor activity in several tumor systems. In particular, compounds 1,5-Bis[[2-(hydroxyethyl)amino]ethyl] aminoanthraquinone **6** and 1,5-Bis-(γ-aminopropyl) aminoanthraquinone **10** were found to possess potent telomerase inhibitory activity at the concentrations of 0.2 and 0.5 μM. Compound **6** was found to possess potent growth inhibitory activity against the human hepatoma G2 cells (IC_{50} 1.2 μM), the rat glioma C6 cells (IC_{50} 1.17 μM) and hepatitis B virus transfected hepatoma cells (HepG 2.2.15, IC_{50} 7.01 μM).[68] Compound **10** was also found to possess potent growth inhibitory activity against the human hepatoma G2 cells (IC_{50} 11.67 μM), the rat glioma C6 cells (IC_{50} 12.18 μM) and hepatitis B virus transfected hepatoma cells (HepG 2.2.15, IC_{50} 7.48 μM).[68] Compound **6** showed distinct advantages over compound **10** in several tumor systems and is tenfold more potent with respect to effective dose range. It is worth noting that the side chain of compound **6** is identical to that of mitoxantrone (Chart 1). The [2-[(2-hydroxyethyl)amino]ethyl]amino side chain might be superior to those containing the secondary and tertiary amino side chain and amido side chain in antineoplastic activity of anthraquinones. However, it appears that the presence of the [2-[(2-hydroxyethyl)amino]ethyl]amino chain is an important, but not a sufficient, factor for good antineoplastic activity, as indicated by the lack of significant biological activity of other ring systems containing this side chain.

We have previously reported the activation of hTERT expression in normal human cells by 1,5-bisthio-substituted or 1,5-bisacycloxy anthraquinones.[11] We also observe here several of 1,5-diaminoanthraquinones that affect hTERT expression in our system. Thus, the bis-substitutions at 1 and 5 positions by thio-, acyloxy-, or amino-group do not appear to affect this activity. Since most of the 1,4-diamidoanthraquinones did not affect hTERT expression in this system, the substitution at 1,5-positions appears to be critical for their activities. Thus, even though it is not apparent what types of substitutions on anthraquinones are crucial for their activity on hTERT expression, the results indicate that the tricyclic anthraquinone structure motif itself might contribute to the activity. This is in sharp contrast to our previous finding that none of the the 1,5-bisthio-substituted or 1,5-bisacycloxy anthraquinones affects telomerase activity. Several of the 1,5-diaminoanthraquinones inhibit telomerase activity, suggesting that an amino-group is important for telomerase inhibitory activity. Among these 1,5-diaminoanthraquinones, substitutions with amines, ether primary or tertiary, appear to be important for its telomerase inhibitory activity, in contrast to the fact that most of the 1,4-diamidoanthraquinones did not affect telomerase activity. Compound **23** has a tertiary amine substitution which might contribute to its inhibitory effect. It is also interesting to note that at least two carbons are required to space amine in the substitutions since compounds **22**, **24**, and **25** with one carbon did not affect telomerase activity. For instance,

the 2,6-diamidoanthraquinones that inhibit telomerase activity also has substitution with two carbons followed by tertiary amine.[56] This structural feature might be crucial for the inhibitory effect.

4. Experimental

4.1. Materials

Melting points were determined with a Büchi 545 melting point apparatus and are uncorrected. ^{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 a Finnigan MAT TSQ-700. The following anthraquinones were prepared by previously described procedures: **2**–**17**[68] and **19**–**55**.[69] All other compounds were commercial materials.

4.2. Cell culture and assessment of hTERT

Non-small 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)[70] 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 three days. To establish stable cell lines in which 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 sub-cloned upstream to a secreted alkaline phosphatase gene (SEAP) and then 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 were similar to their parental cells.

4.3. 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.[71,72] 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 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 various designated concentrations. After 48 h, 25 μl of MTT were added to each well and 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.

4.4. Telomerase assay

A modified telomeric repeat amplification protocol (TRAP-G4) was utilized for G-quadruplex-induced telomerase activity assay.[57] The telomerase products were resolved by 10% polyacrylamide gel electrophoresis and visualized by staining with SYBER Green. As a source for telomerase, the total cell lysates derived from lung cancer cell line H1299 cells were used. Protein concentration of the lysates was assayed using a Bio-Rad protein assay kit according to BSA standards.

4.5. SEAP assay[73]

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, 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. Equal amounts of SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, and 20 mM L-homoarginine) was added to the media, and *p*-nitrophenylphosphate was added to a final concentration of 12 mM. Absorptions at 405 nm were taken and the rate of absorption increase was determined.

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