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Synthesis, human telomerase inhibition and anti-proliferative studies of a series of 2,7-bis-substituted amido-anthraquinone derivatives

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ABSTRACT

Telomerase is important in tumor initiation and cellular immortalization. Given the striking correlations between telomerase activity and proliferation capacity in tumor cells, telomerase had been considered as a potentially important molecular target in cancer therapeutics. A series of 2,7-diamidoanthraquinone were designed and synthesized. They were evaluated for their effects on telomerase activity, hTERT expression, cell proliferations, and cytotoxicity. In the series, compounds (**6**, **10**, **13**, **16**, **18**, **19**, **20**–**22**, and **24**) showed potent telomerase inhibitory activity, while compounds **19**, **21**, and **22** activated hTERT expression in normal human fibroblasts. The results indicated that 2,7-diamidoanthraquinones represent an important class of compounds for telomerase-related drug developments. Compounds **8**, **16**, **18**, **26**, and **32** were also selected by the NCI for Screening Program and demonstrated high anti-proliferative activity against 60 human cancer cell lines. Structure–activity relationships (SAR) study revealed that the test compounds with side chains two carbon spacer between amido and amine are important structural moiety for telomerase inhibition. Although the exact mechanism of how this amine group contributes to its activity is still unclear, however, the amine group in the extended arm of the bis-substituted anthraquinone might contribute to proper binding to the residues within the grove of G-quadruplex structure. Our results indicated that the 2,7-disubstituted amido-anthraquinones are potent telomerase inhibitors that have the potential to be further developed into novel anticancer chemotherapeutic agents.

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] Telomerase is a reverse transcriptase that prevents the mortality checkpoints evoked by programmed telomere shortening during each round of cell division.[2] Expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, appears to be a key determinant for telomerase activity. Insufficient hTERT expression in most mortal somatic cells cannot produce enough telomerase to maintain telomere length during cycles of chromosome replication.[3,4] The hTERT is highly expressed approximately 90% in stem cells, germ cell lines, and most human tumors, and its inhibition represents a strategy for the development of selective anti-cancer drugs.[5–9] It is also well accepted that human cancer cells achieve immortalization in large part through the illegitimate activation of telomerase expression. The reactivation of telomerase activity in most cancer cells supports the concept that telomerase is a relevant target in oncology, and telomerase inhibitors have been proposed as new potential anti-cancer agents.[10] The important roles of telomerase in tumor initiation and cellular immortalization have led to the identification of telomerase as a potentially important molecular target in cancer therapeutics. The development of telomerase inhibitors and G-quadruplex stabilizers has emerged as a highly promising approach.[11–14]

Dye ligands have been considered as one of the important alternatives to natural counterparts for binding most types of proteins, because they interact with the active sites of many proteins mimicking the structure of the substrates, cofactors, or binding agents for those proteins.[15–17] Interactions between the dye ligand and proteins can be achieved by complex combination of electrostatic, hydrophobic, hydrogen bonding. Most of these reactive dyes consist of a chromophore (either azo dyes, anthraquinone, or phathalocyanine), linked to a reactive group (often a mono- or dichlorotriazine ring).[18] Anthraquinone-containing extracts from different plant sources such as senna, cascara, aloe, frangula, and rhubarb have been found to have wide variety of pharmacological

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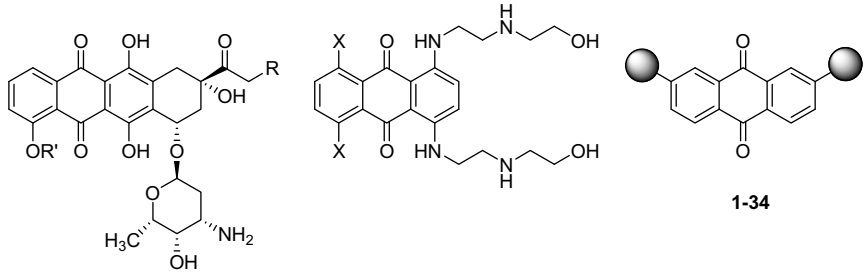


Chart 1. Structures of bioactive chromophoric anthraquinones.

activities such as antiinflammatory, wound healing, analgesic, antipyretic, antimicrobial, and antitumor activities.[19] Furthermore, anthraquinones, porphyrins, ethidium bromide derivatives, fluorenones, heteroaromatic polycyclic systems, quinolines, acridines, and telomestatins have been identified with high affinity and selectivity for human quadruplex telomeric sequence.[14,20,21] Several of the anthraquinone derivatives were also been used as chemotherapeutic agents in anticancer treatments (see Chart 1). The anthracycline antibiotics (daunorubicin and doxorubicin) have been introduced in clinical use more than 30 years ago for treatment of wide variety of cancers.[22] Mitoxantrone and ametantrone are another member of 1,4-disubstituted anthraquinones family developed to treat malignancies but has rather narrow spectrum of antitumor activities that limits their clinical usage. They are synthetic representatives of a group of compounds termed aminoanthraquinones.[23]

It was first reported by Neidle and co-workers that anthraquinones are potent human telomerase inhibitors.[24–28] Previous work by their laboratory had demonstrated that disubstituted anthraquinones with progressively increasingly bulky substituents have close relationship between telomerase inhibition and binding energy for the G-quadruplex structure form by human telomeric DNA sequences.[29] To date, a number of families of compounds have been developed and their telomerase activity and cytotoxicity have been extensively studied.[30–33] It was also shown that the planar moieties of anthraquinones bind onto the end of the G-quadruplex structure through π–π stacking interactions with the guanine residues and the substituents bind to the grooves of the G-quadruplex structures.[24,26,30,34–36] Among the quadruplex ligands studies, disubstituted amido-anthraquinones represent one of the best small molecules of how the pattern of disubstitution modulates DNA duplex versus quadruplex selectivity.[24,26,37] Thus, anthraquinones might inhibit telomerase-dependent telomere elongation in vitro by stabilizing of G-quadruplex structures by sequestering the DNA substrate and making it inaccessible to the telomerase.[38]

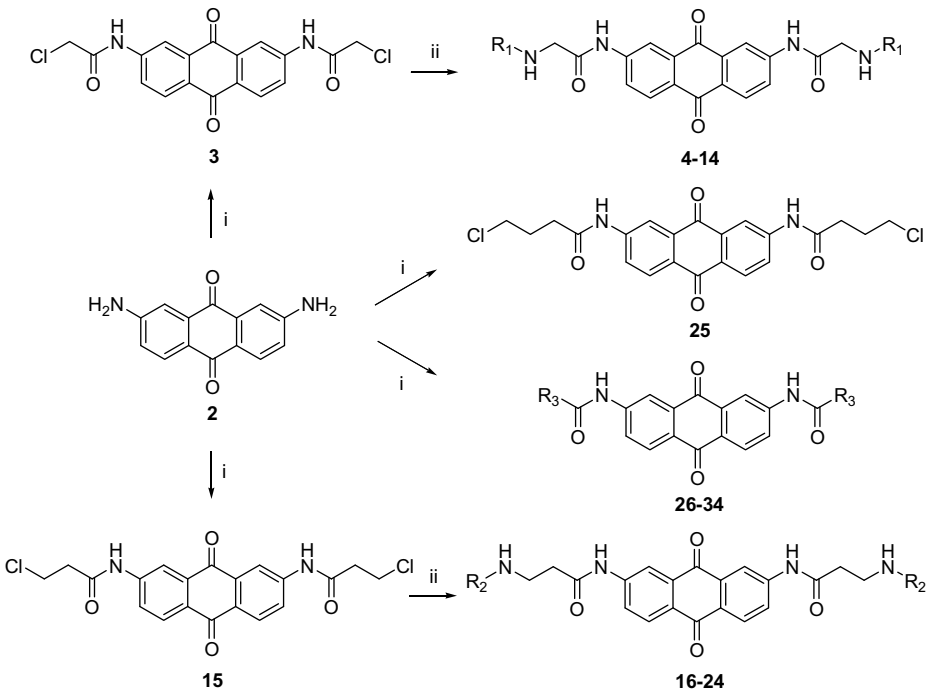
To explore novel and potent telomerase inhibitors, we have recently synthesized a series of anthraquinones and examines their toxicity to cancer cells as well as their telomerase inhibitory activity. In past work we have reported the synthesis of a range of structurally diverse anthraquinones.[32,33,39–47] In the present study, we have designed and synthesized a series of 2,7-disubstituted amidoanthraquinone derivatives and evaluated their effects to telomerase activity as inferred from the TRAP assay and collated some selected compounds growth-percent against tumor cell lines in vitro (NCI60 assays). The antiproliferative effect and hTERT repressing activity of these newly synthesized compounds were also determined. Our results indicated that the 2,7-disubstituted amido-anthraquinones are potent telomerase inhibitors that have the potential to be further developed to be novel anticancer chemotherapeutic agents.

2. Chemistry

In previous papers in this series we have described a range of synthetic route to, biological properties of anthraquinones generally symmetrical substituted in the 1,4-, 1,5-, 1,8-, 2,6-positions including benzo-annelation, as well as their mono substituted derivatives.[32,33,39–47] The backbone of these compounds resembles some of the anti-cancer agents mitoxantrone and ametantrone, and were prepared by two-stage reactions which are similar to our previous papers.[32,44,46] Our strategy for achieving this objective is symmetric synthesis of 2,7-diamidoanthraquinone derivatives and a number of alkylacyl chlorides for acylation of aromatic amine **2** via N-acylation have been synthesized. After considerable effort we were able to develop a process by which a 2,7-disubstituted anthraquinones can be synthesized and the preparation involved two-step synthetic route with appropriate yields (overall 20–74% in three steps) (Scheme 1): (1) acylation reaction of 2,7-diaminoanthraquinone **2** with various alkylacyl chlorides yielded the corresponding 2,7-bis-(ω-chloroacylamido) side chain compounds **3** and **15**; (2) followed by nucleophilic displacement of the chloride with primary or secondary amines to produce symmetrical peptoid dimer analogs **4**–**14**, and **16**–**24**, respectively; (3) compounds **26**–**34** were synthesized by direct one-step acylation reaction using acid chlorides to obtain various amido-substituted derivatives. The chloroacetamido- and chloropropionami-do-substituted (**3 and 15**) were then converted to the aliphatic amido-anthraquinones (**4**–**14 16**–**24**) upon heating in mini-reactor at 130 °C. However, under these reaction conditions significant amounts of the reaction products were isolated along with the desired reaction products. The quantity of the byproducts isolated was substrate dependant and purification of the reaction mixtures required tedious recrystallization and chromatography. Under these conditions, a more effective acylation catalyst can achieve the desired amide linker derivatives (**26**–**34**) with appropriate yields.

3. Telomerase activity

We evaluate the effects of these 2,7-diamidoanthraquinones on telomerase inhibition using PCR-based telomerase assay, and TRAP (telomeric repeat amplification protocol) assay. We also designed and expressed SEAP assay, MTT assay, and TRAP assay to provide a suitable target for telomerase activity assay. Results are summarized in Table 1 and all test derivatives showed MTT and SEAP inhibition activity in micromolar range. This strategy takes advantage of the anthraquinone-base structure for analysis. A related construct was used as a telomerase inhibitor, in studies mentioned above. Given the striking correlations between telomerase activity and anti-proliferation capacity in tumor cells, we expected that analysis of anthraquinone-base chromophore telomerase might



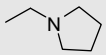
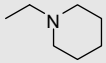
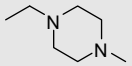
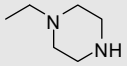
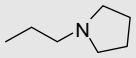
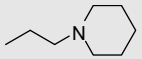
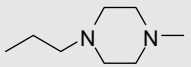
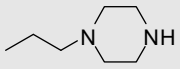
Scheme 1. Reagents and conditions: (i) RCOCl, DMF, pyridine, rt, 24 h; (ii) RNH_2, pyridine, mini-reactor, 130 °C, 40 min.

yield further insight into this relationship. We screened a series of anthraquinone-base derivatives, searching for compounds that exhibit inhibition of telomerase in SEAP assay and less cytotoxicity in MTT assay. As shown in Table 1, compound **3**, which contains simple side chain of $-CH_2Cl$ unit appended to the substituent in the 2,7-positions, showed high selective inhibition activity toward H1299 cancer cells over hTERT-immortalized normal cells. Therefore, the torsion angles of various compounds substituted on the anthraquinone might be related to the inhibitory activity on receptor binding site. As an alternative possibility, the substituent at the appropriate position might fill a cavity of the receptor site. The results of a structure–activity relationships (SAR) study indicated that the torsion angle between the central chromophore ring and the chloroacetamido substituent, and the bond length of linker at the anthraquinone moiety, might be important for G-quadruplex binding activity. We also evaluate the effects of these disubstituted 2,7-amidoanthraquinones on telomerase activity using PCR-based telomerase assay as well as TRAP assay. Comparative in vitro telomerase inhibitory screening of this group of compounds provided some intriguing observations of inhibitory profiles. At 1–20 μM concentrations, among a total of 2,7-diamidoanthraquinones tested, we found 10 of them showed telomerase inhibitory effect. The inhibitory effects of these compounds on telomerase are specific as they did not affect the internal controls (ICs) for Taq polymerase in our assays (Fig. 1). Among a total of compounds tested, several trends were also evident from the results of testing this group of compounds, we found that seven compounds (**16**, **18**–**19, 20, 21, 22**, and **24**) showed telomerase inhibitory effect at the concentrations of 1, 5, 10, and 20 μM. These seven compounds of 2,7-diamidoanthraquinones with side chains two carbon spacer between amido and amine. It is interesting to note that most of these compounds with side chain $-CH_2CH_2N-$ did affect telomerase inhibitory activity. Thus, in addition to anti-cancer function, our finding indicated that the spacer and the nature of the center nitrogen atom of the side chain might have potential SAR for telomerase inhibitory activity and antineoplastic activity. As shown in Figure 1, compounds with side chains $-NHCO-CH_2NHC_2H_5$ **10**, $-NHCO-(CH_2)_2NHC_3H_7$ **21,** and $-NHCO-(CH_2)_2NHC_2H_5$ **22** show inhibition of G-quadruplex-induced telomerase at the IC_{50} levels of 16.0, 13.3, and 9.7 μM, respectively. Compound with side chain pyrrole **18** (IC_{50} 6.7 μM) and piperidine **19** (IC_{50} 8.4 μM) is the most potent telomerase inhibitors in this series of compounds. As shown in Figure 2, the SEAP activities are elevated in cells that are comparison with MTT and SEAP. Compounds with side chains $-NHCO-(CH_2)_2NC_5H_{10}$ **19**, $-NHCO-(CH_2)_2NHC_3H_7$ **21**, and $-NHCO-(CH_2)_2NHC_2H_5$ **22** show significant activity of P_{hTERT}-SEAP.

4. Antitumor activity

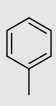
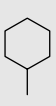
The 2,7-disubstituted anthraquinone derivatives were studied for anti-proliferative activity against human cancer cell lines in National Cancer Institute Drug Screen Program. Out of the synthesized derivatives, compounds **8, 16**, **18**, **26**, and **32** have been selected by NCI. Those potential compounds were evaluated in the full panel of human tumor cell lines derived from nine cancer cell types (non-small cell lung cancer, colon cancer, breast cancer, ovarian cancer, leukemia, renal cancer, melanoma, prostate cancer, CNS cancer). They were evaluated in the 60 cell line panel and anticancer assay was performed in accordance with the protocol of developmental therapeutics program (one dose mean graph). Results for each compound were reported as the growth percentage of the treated cells when compared to that of the untreated control cells. Our results indicated that only compounds **8**, **16**, and **18** showed significant cytotoxic activity. The growth percent values at 10^{-5} molar for 60 cancer cell lines are shown in Table 2. It is especially notable that compound **8** with side chain $-NHCO-CH_2N(CH_3)_2$ displayed relatively potent and differential cytotoxic activity. The colon cancer panel, leukemia, renal, melanoma and CNS cancer lines were quite sensitive to **8**, while the non-small cell lung cancer lines, breast, ovarian and prostate panels were less sensitive. It is surprising since compound **16** with side chain $-NHCO-(CH_2)_2N(C_2H_5)_2$ has similar differences in cytotoxicity which might have a significant effect on their interaction with DNA. It is especially notable that compound **16** had both cytotoxicity and telomerase activity whereas for cytocidal effects this difference was significant. However, it had significant influence on

Table 1
Effects of symmetrical 2,7-diamidoanthraquinone analogs on activating or repressing hTERT expression and telomerase activity

Compound	R (R_1, R_2, R_3)	Cell type (inhibition μM ± SD)[a]											
		H1299						BJ1					
		MTT			SEAP			MTT			SEAP		
		100	10	1	100	10	1	100	10	1	100	10	1
1	2,7-Dinitroanthraquinone	69 ± 10	95 ± 8	97 ± 15	62 ± 8	85 ± 21	87 ± 13	86 ± 3	91 ± 2	91 ± 2	65 ± 23	60 ± 35	55 ± 21
2	2,7-Diaminoanthraquinone	65 ± 10	80 ± 16	90 ± 17	63 ± 12	75 ± 8	87 ± 10	80 ± 2	83 ± 1	95 ± 2	ND	ND	ND
3	CH_2Cl	1 ± 0	5 ± 3	58 ± 44	36 ± 1	40 ± 1	72 ± 3	88 ± 2	89 ± 3	105 ± 2	135 ± 21	69 ± 34	ND
4	$CH_2N(CH_2CH_3)_2$	25 ± 3	47 ± 7	101 ± 8	37 ± 10	61 ± 11	90 ± 3	50 ± 1	67 ± 2	116 ± 4	116 ± 34	131 ± 57	26 ± 4
5	$CH_2NHCH_2CH_2CH_2CH_3$	15 ± 11	17 ± 9	91 ± 16	20 ± 13	26 ± 17	77 ± 18	43 ± 1	41 ± 1	103 ± 1	80 ± 14	24 ± 24	27 ± 18
6		23 ± 14	52 ± 13	99 ± 9	30 ± 10	48 ± 13	88 ± 10	39 ± 1	60 ± 2	99 ± 2	ND	ND	ND
7		35 ± 11	37 ± 15	50 ± 16	63 ± 15	69 ± 20	74 ± 19	22 ± 2	78 ± 3	89 ± 1	103 ± 5	121 ± 3	118 ± 16
8	$CH_2N(CH_3)_2$	15 ± 12	55 ± 9	92 ± 13	21 ± 14	40 ± 20	87 ± 12	39 ± 1	45 ± 1	96 ± 3	3 ± 13	44 ± 5	96 ± 1
9	$CH_2NHCH_2CH_2CH_3$	14 ± 10	54 ± 11	89 ± 15	24 ± 10	44 ± 16	73 ± 17	42 ± 1	44 ± 0	96 ± 3	ND	ND	ND
10	$CH_2NHCH_2CH_3$	3 ± 4	24 ± 5	52 ± 11	34 ± 3	35 ± 5	60 ± 10	37 ± 1	56 ± 2	72 ± 2	17 ± 21	41 ± 4	90 ± 5
11		37 ± 6	71 ± 4	90 ± 14	23 ± 9	50 ± 11	76 ± 21	47 ± 3	69 ± 1	86 ± 1	97 ± 30	68 ± 21	61 ± 21
12		88 ± 11	85 ± 13	88 ± 19	60 ± 12	72 ± 20	89 ± 17	76 ± 2	85 ± 15	88 ± 2	ND	ND	ND
13	$CH_2NHCH(CH_3)_2$	6 ± 11	26 ± 15	78 ± 10	21 ± 16	28 ± 18	73 ± 31	39 ± 1	41 ± 2	89 ± 1	45 ± 11	55 ± 19	79 ± 14
14	$CH_2NHCH_2CH(CH_3)_2$	11 ± 5	39 ± 10	108 ± 8	26 ± 10	38 ± 15	73 ± 20	39 ± 0	37 ± 0	107 ± 3	40 ± 3	44 ± 2	83 ± 5
15	CH_2CH_2Cl	29 ± 11	44 ± 15	77 ± 13	87 ± 9	94 ± 7	94 ± 11	39 ± 1	65 ± 1	77 ± 2	ND	ND	ND
16	$CH_2CH_2N(CH_2CH_3)_2$	1 ± 1	41 ± 20	106 ± 15	26 ± 11	35 ± 17	76 ± 19	36 ± 1	55 ± 3	103 ± 2	37 ± 5	49 ± 3	84 ± 5
17	$CH_2CH_2NHCH_2CH_2CH_2CH_3$	14 ± 10	52 ± 4	82 ± 12	22 ± 17	33 ± 9	56 ± 14	40 ± 0	70 ± 1	80 ± 3	111 ± 25	95 ± 46	88 ± 34
18		12 ± 10	54 ± 7	87 ± 15	17 ± 13	29 ± 15	73 ± 11	39 ± 0	68 ± 0	82 ± 4	109 ± 18	67 ± 16	ND
19		18 ± 9	44 ± 7	87 ± 15	25 ± 10	33 ± 8	55 ± 23	41 ± 2	54 ± 2	92 ± 6	234 ± 20	226 ± 51	186 ± 47
20	$CH_2CH_2N(CH_3)_2$	1 ± 1	53 ± 13	1 ± 104	1 ± 22	13 ± 30	5 ± 94	22 ± 13	30 ± 14	94 ± 7	38 ± 0	61 ± 2	86 ± 2
21	$CH_2CH_2NHCH_2CH_2CH_3$	13 ± 10	50 ± 6	80 ± 15	13 ± 15	22 ± 14	52 ± 13	38 ± 2	65 ± 2	75 ± 1	217 ± 40	133 ± 48	ND
22	$CH_2CH_2NHCH_2CH_3$	34 ± 7	59 ± 3	86 ± 10	22 ± 13	33 ± 24	68 ± 29	42 ± 1	73 ± 3	82 ± 2	97 ± 87	217 ± 133	211 ± 38
23		39 ± 6	80 ± 15	95 ± 18	21 ± 10	52 ± 11	75 ± 16	61 ± 1	80 ± 2	95 ± 2	232 ± 66	138 ± 115	ND
24		102 ± 13	94 ± 8	97 ± 8	71 ± 15	71 ± 21	75 ± 26	88 ± 4	100 ± 3	96 ± 2	131 ± 29	118 ± 26	163 ± 22
25	$CH_2CH_2CH_2Cl$	22 ± 2	40 ± 2	84 ± 18	78 ± 17	86 ± 13	88 ± 19	58 ± 3	62 ± 2	72 ± 3	75 ± 16	42 ± 45	13 ± 58
26	CH_3	55 ± 11	83 ± 13	117 ± 15	74 ± 18	87 ± 14	97 ± 10	91 ± 2	91 ± 2	106 ± 1	117 ± 5	117 ± 3	123 ± 5
27	CH_2CH_3	35 ± 15	38 ± 17	70 ± 25	85 ± 16	95 ± 14	90 ± 23	48 ± 2	57 ± 2	77 ± 2	51 ± 8	64 ± 8	78 ± 10
28	$CH_2CH_2CH_3$	40 ± 15	59 ± 13	93 ± 14	83 ± 4	83 ± 6	90 ± 8	50 ± 6	51 ± 8	84 ± 2	ND	ND	ND

(*continued on next page*)

Table 1 (*continued*)

Compound	R (R_1, R_2, R_3)	Cell type (inhibition μM ± SD)[a]											
		H1299						BJ1					
		MTT			SEAP			MTT			SEAP		
		100	10	1	100	10	1	100	10	1	100	10	1
29		41 ± 30	47 ± 13	71 ± 24	85 ± 19	92 ± 11	104 ± 10	97 ± 3	85 ± 2	92 ± 2	36 ± 13	72 ± 8	89 ± 2
30		75 ± 22	96 ± 17	106 ± 12	80 ± 17	91 ± 21	97 ± 18	86 ± 4	80 ± 3	93 ± 2	40 ± 15	79 ± 6	85 ± 8
31		76 ± 18	94 ± 13	102 ± 11	61 ± 13	79 ± 8	89 ± 23	44 ± 1	44 ± 0	89 ± 2	43 ± 75	92 ± 45	74 ± 29
32		43 ± 15	36 ± 13	46 ± 14	81 ± 1	81 ± 1	80 ± 1	47 ± 2	44 ± 10	60 ± 6	ND	ND	ND
33		42 ± 12	77 ± 6	91 ± 12	85 ± 13	87 ± 2	83 ± 5	58 ± 1	68 ± 1	77 ± 1	108 ± 15	130 ± 15	137 ± 15
34		52 ± 16	63 ± 13	90 ± 3	80 ± 2	93 ± 3	96 ± 5	64 ± 2	76 ± 3	87 ± 2	22 ± 7	14 ± 53	14 ± 67

ND, not determined.

[a] SD, standard derivation, all experiments were independently performed at least three times.

the spectrum of anti-proliferative activity. Compound **18** had selective cytotoxicity for colon and melanoma cell lines, and compounds **26** and **32** did not have significant growth inhibitory activities. The lack of anti-proliferative activity of compounds **26** and **32** demonstrates the important role of the spacer length between the amino group and chromophore for cytotoxic activity.[48] The optimal number of atoms between amido nitrogen atom and terminal side chain nitrogen is 2, which corresponds to ~4 Å distance. NCI60 cell line panel assays from some selective compounds give only limited information and observations on mechanisms of drug action; for these DNA-affinic tricyclic ring systems more precise physicochemical measurements are required which DNA polymorphic form is the target.

5. Results and discussion

At present, we described a method of synthesizing 2,7 bis-substituted anthraquinones and compared to their cytotoxicity and telomerase activity. A structure–activity relationship study was also conducted on a number of amidoanthraquinone derivatives.[32,33,41,45,46,49–53] Appropriately substituted amido-anthraquinones are effective G-quadruplex stabilizers, but without sufficient information is available as yet on the possible modification of G-quadruplex recognition and telomerase inhibition produced by the position of the amide bond.[31] In this investigation, we continue to focus our attention on the role of our systematic synthesized 2,7-diamidoanthraquinones bearing the –CO–NH– group and spacer linked to the planar anthraquinone moiety and to understand the basis of amido-anthraquinones selectivity. The chemical and biological activities of anthraquinone compounds are greatly affected by its various substituents of the planar tricyclic ring system which considered as aglycon analogs of anthracycline antibiotics. 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. The structure–activity analysis of these compounds also revealed that a terminal long aliphatic chain is not a favorable inhibitor for telomerase (compounds **5**, **17**). In contrast, a cyclic or branched terminal chain might favor telomerase inhibition.

The telomerase activity is regulated mostly at the transcriptional level for its catalytic subunit, *hTERT*, and partly at the post-translational level.[54] Since the expression of human telomerase catalytic component is the key regulator in telomerase activity, we analyzed the expression of telomerase by monitoring the expression of *hTERT* as the criteria. To facilitate the analysis, we constructed a reporter system that enables the evaluation of the *hTERT* promoter activity using a reporter gene, SEAP, in cancer cell line H1299 and *hTERT*-immortalized normal cell line *hTERT*-BJ1. 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. As shown in Table 1, most of the compounds did show various effects on SEAP expression. However, all of them 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 inhibits cell proliferation.

We then used the expression of SEAP in hTERT-BJ1 cells harboring P_{hTERT}-SEAP as the criteria 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 Table 1. The cytotoxicity levels in

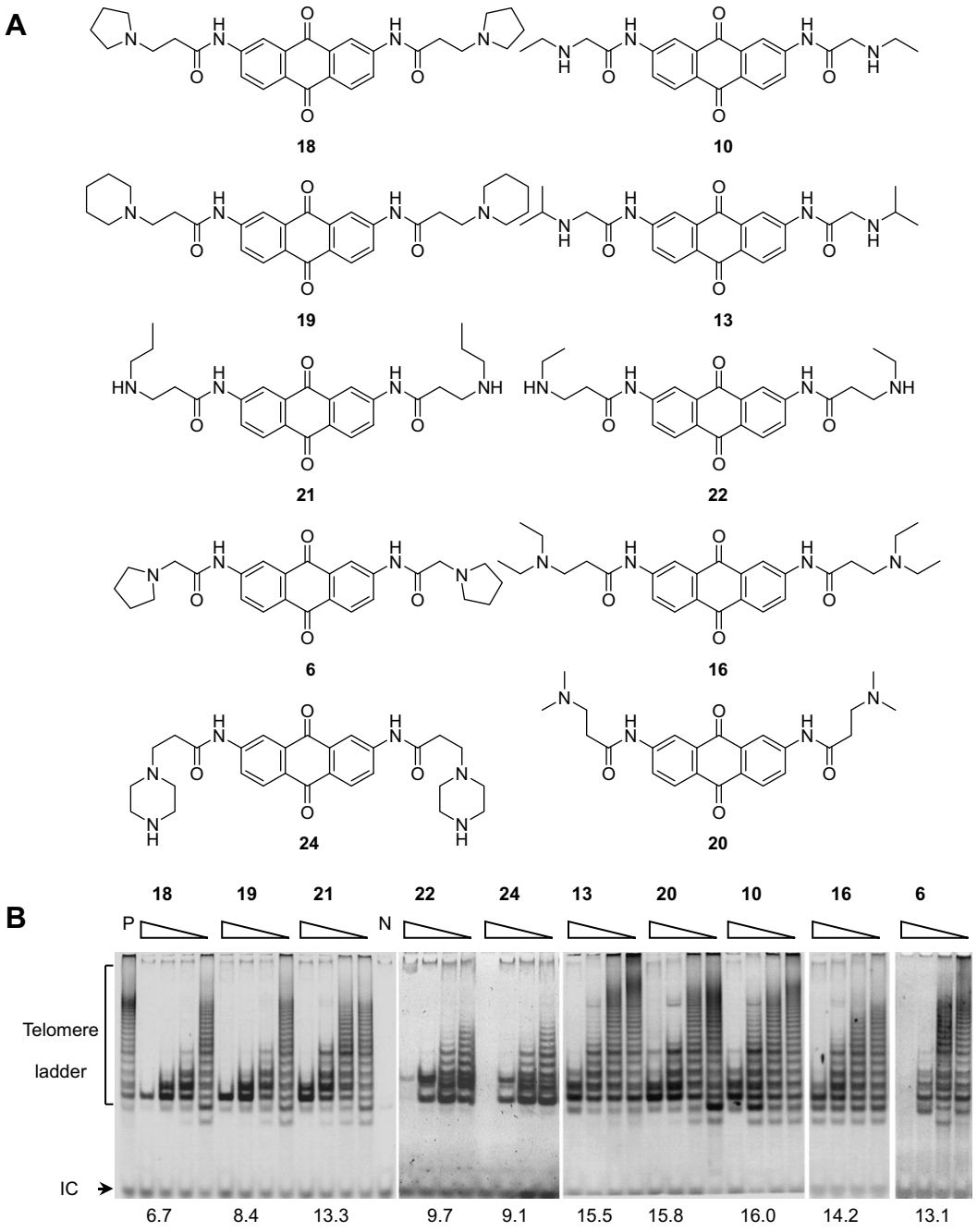


Figure 1. Inhibition of telomerase activity by symmetrical 2,7-diamidoanthraquinone. (A) Structure of 2,7-diamidoanthraquinones that showed telomerase inhibitory activities. (B) TRAP assay was conducted using cell extracts prepared from H1299 cells and 2 μg of extracts were used in each assay. Extended 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 was 1, 5, 10, 20 μM, respectively. Because the internal control (IC) shares one oligonucleotide with the reaction, the IC products became apparent when telomerase activities were inhibited. P is positive control and N is negative control.

hTERT-BJ1 cells are moderately higher than that in human tumor cell lines for most of the tested compounds. Strikingly, compound **3** showed high selective inhibiting activity toward H1299 cancer cells over *hTERT*-immortalized normal cells (Table 1). Although the mechanism of preferential inhibition of compound **3** is still unclear to us, the compound might has the potential to be developed into a drug for anticancer therapies. We also found several 2,7-diamidoanthraquinone derivatives activated the SEAP levels (compounds **19**, **21**, **22**). Interestingly, these three compounds all carry R = CH_2CH_2N– groups, suggesting that the structural moiety might be important for its activity in activate hTERT expression. Among these compounds, we are most interested in compound **22** because it activated the SEAP levels without greatly affecting the cell viability at low concentrations (Fig. 2).

6. Conclusion

In summary, we have designed and synthesized a series of 2,7-diamidoanthraquinones and evaluated their effects on telomerase activity, hTERT expression, cell proliferations. Given the striking correlations between telomerase activity and proliferation capacity in tumor cells, we expected that analysis of anthraquinone-base chromophore telomerase might yield further insight into designing better compounds for anticancer therapies. Of the series of com-

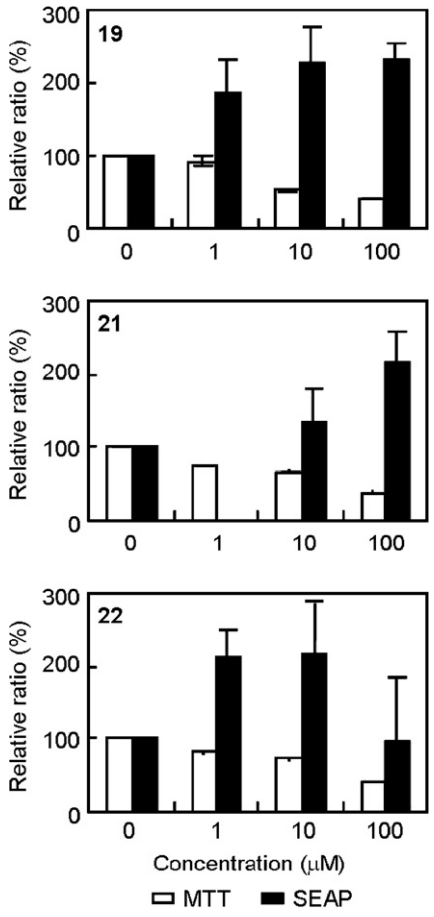


Figure 2. Activation of P_{hTERT}-SEAP by 2,7-diamidoanthraquinone. About 2×10^3 cells of hTERT-BJ1 harboring P_{hTERT}-SEAP or P_{CMV}-SEAP were seeded in 96-well plates and incubated at 37 °C for 24 h. Cells were then washed with PBS, recultured in fresh media, and incubated with varying amounts of compounds **19**, **21**, or **22** 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. Values were presented from six experiments using the values without drug treatment as 100%.

pounds analyzed, compounds (**6**, **10**, **13**, **16**, **18**–**22** and **24**) showed potent telomerase inhibitory activity, compounds (**19**, **21** and **22**) activated hTERT expression in normal human fibroblasts. The results indicated that 2,7-diamidoanthraquinones represent an important class of compounds for telomerase-related drug developments. The results of structure–activity relationships (SAR) study also revealed that an amine with two carbon space is an important structural moiety for telomerase inhibition. The exact mechanism of how this amine group contributes to its activity is unclear. However, the amine group in the extended arm of the disubstituted anthraquinone might contribute to proper binding to the residues within the grove of G-quadruplex structure. The number of C might determine the length of the extended arm for its proper bindings.

The mechanism of how 2,7-diamidoanthraquinones affect hTERT expression and behind these diversified biological effects is still unclear. Since several of anthraquinone derivatives were shown to have G-quadruplex stabilizing activity, it is possible that compounds **19**, **21**,**22** affect hTERT expression through stabilizing of G-quadruplex structure in the promoter region. Indeed, stabilization of G-quadruplex structures by small molecule compounds was shown to repress the expression of *c-myc*.[55] A bioinformatic survey of hTERT promoter for putative G-quadruplex-forming sequences has identified one such sequence located at −2963

Table 2
Cytotoxicity of symmetrical 2,7-diamidoanthraquinone derivatives in the NCI in vitro 60-cell drug screen program

Growth percent[a] (at 10^{-5} M)	Compound				
	8	**16**	**18**	**26**	**32**
Non-small cell lung cancer					
EKVX	102.30	59.99	58.72	95.28	94.31
HOP-62	28.97	11.65	18.79	110.60	91.64
HOP-92	56.14	9.70	7.04	101.26	86.18
NCI-H226	89.14	49.89	41.10	104.51	100.60
NCI-H23	51.78	30.34	31.49	94.24	95.85
NCI-H322M	32.42	44.75	45.79	97.50	105.77
NCI-H460	5.01	9.50	25.52	109.29	89.03
NCI-H522	61.82	27.98	29.16	93.56	89.41
Colon cancer					
COLO 205	−73.57	−49.69	−29.04	119.85	91.92
HCC-2998	−73.97	−87.42	19.99	105.35	132.37
HCT-116	19.07	−32.45	10.51	101.91	87.14
HCT-15	16.26	17.42	56.14	93.93	86.35
HT29	11.73	3.30	19.47	107.23	106.26
KM12	66.95	21.82	32.55	105.94	92.67
SW-620	23.36	16.93	20.01	103.21	104.75
Breast cancer					
BT-549	85.36	57.04	76.91	93.29	92.51
HS 578T	−6.11	−23.77	5.26	133.05	118.90
MCF7	7.92	9.34	13.35	96.90	77.38
MDA-MB-231/ATCC	4.49	31.32	30.47	103.99	97.05
MDA-MB-435	68.36	71.07	68.84	109.48	97.35
NCI/ADR-RES	47.84	59.52	80.59	114.26	90.83
T-47D	24.62	68.62	58.98	96.17	79.75
Ovarian cancer					
OVCAR-3	43.63	10.39	36.88	111.06	103.35
OVCAR-4	45.70	24.92	49.82	103.60	97.54
OVCAR-5	73.15	33.63	18.81	102.27	112.01
OVCAR-8	32.31	4.30	19.58	99.85	94.34
SK-OV-3	70.26	44.02	27.14	101.12	93.16
Leukemia					
CCRF-CEM	−13.21	−6.02	3.74	99.77	90.67
HL-60(TB)	−64.97	14.01	85.84	38.74	62.45
K-562	34.91	8.10	6.40	98.54	91.60
MOLT-4	29.12	5.42	22.97	96.96	94.29
RPMI-8226	34.97	−29.02	47.90	121.47	88.38
SR	-46.45	−0.39	19.75	3.61	1.69
Renal cancer					
786-0	68.60	24.72	40.51	97.72	94.92
A498	−6.39	−38.76	−0.91	115.60	92.35
ACHN	66.43	11.09	29.65	96.93	86.70
CAK1-1	59.26	3.27	55.55	105.10	88.43
SN12C	27.99	8.63	6.68	113.35	82.93
TK-10	73.47	17.16	31.22	118.38	136.20
UO-31	−82.55	−31.53	5.27	96.42	95.55
Melanoma					
LOX IMVI	−52.34	3.39	30.74	99.05	90.27
M14	75.04	62.79	53.88	105.11	98.89
MALME-3M	96.21	62.39	47.00	111.26	91.76
SK-MEL-2	−30.35	−60.38	−45.21	30.15	16.85
SK-MEL-28	32.11	2.56	14.99	117.60	108.51
SK-MEL-5	82.59	82.75	56.94	108.87	75.67
UACC-257	−1.84	−14.46	−17.88	26.97	107.63
UACC-62	22.31	44.45	42.72	101.24	81.00
Prostate cancer					
DU-145	78.68	10.20	29.53	116.79	98.83
PC-3	59.00	13.59	21.66	102.73	106.89
CNS cancer					
SF-268	64.92	35.18	26.67	113.68	95.09
SF-295	−83.51	−19.91	48.62	103.25	86.60
SF-539	−3.72	37.15	33.75	103.04	93.24
SNB-19	79.57	75.84	66.97	94.40	98.79
SNB-75	76.12	46.62	77.47	106.91	89.23
U251	16.26	21.65	18.16	95.14	72.62
Mean	28.70	16.33	30.97	99.06	91.54
Delta	112.21	103.75	76.18	95.45	89.85
Range	185.81	170.17	131.05	129.44	134.51

[a] Data obtained from NCI in vitro 60-cell drug screen program at 10^{-5} M concentration.

position. Even though it remains to be tested for the biological relevance of this sequences in hTERT promoter, several of these G-quadruplex-forming sequences are indeed capable of forming G-quadruplex structures in other genes. For example, the G-rich region within the promoter sequences of *c-myc, VEGF, c-kit, BCL2*, and *KRAS* proto-oncogenes was capable of forming G-quadruplex structures.[55–59] It will be interesting to test the role of this putative G-quadruplex-forming sequence in hTERT expression. Anthraquinone-base derivatives are known for their potential antitumor and telomerase activities. Since telomerase reverse transcriptase activity depends on the 3′ single-stranded telomeric DNA end acting as a primer,[31] disubstituted amidoanthraquinone molecules represent an example that might bind and stabilize the folded quadruplex form of the primer and DNA duplex. The aim of this study was to elucidate the structure–activity relationships of simple 2,7-symmetrical bis-substituted analogs of amido-anthraquinone to further delineate the nature of the requirements of the pharmacophore. A striking feature of the results given in Tables 1 and 2 is the marked repressing hTERT expression [P_{hTERT}-SEAP (H1299)], [P_{hTERT}-SEAP (BJ-1)] and cytotoxicity. The inhibitory effect and cytotoxicity do not seem to correlate with the modifying groups. As a whole, these data support the conclusion that all the tested molecules, including those reported here, share a common target in telomerase and cytotoxicity. These results confirm amide bond direction in amido-anthraquinones that would favor G-quadruplex recognition, DNA-interactive, and targeting telomerase in preventive and/or curative therapy.

7. Experimental

7.1. General procedures

Melting points were determined with a Büchi 545 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. Mass spectra (EI, 70 eV, unless otherwise stated) were obtained on a Finnigan MAT TSQ-46 and Finnigan MAT TSQ-700. All other reagents and solvents were purchased from either Aldrich or Merck and used without further purification.

7.2. General synthetic methods

2,7-Diaminoanthraquinone (**2**) were prepared according to the literature.[26] All anthraquinone-base derivatives were synthesized by using a simply acylation or two-stage reaction in mini-reactor. These compounds were obtained in good yield and their purity was determined using mass spectrometry, ^{1}H NMR, and ^{13}C NMR.

7.2.1. 2,7-Bis(chloroacetamido)anthraquinone (3)

2,7-Diaminoanthraquinone (0.238 g, 1 mmol) was dissolved in DMF (20 mL) in ice-bath and pyridine (0.5 mL) and chloroacetyl chloride (0.5 mL, 6 mmol) were added under nitrogen. The mixture was stirred for 24 h. Ice was added to precipitate out the crude product. The resulting precipitate was collected by filtration, washed with diethyl ether, and purified by crystallization from ethanol. Product 3 was obtained as slightly yellow powder (yield 59%): mp 286 °C (EtOH). IR (KBr) (cm^{-1}) 1672, 1718, 3315. MS (EI, 70 eV) = 390.0 (M^+). ^{1}H NMR (300 MHz, DMSO): δ ppm 4.32 (s, 4H), 7.97 (dd, J = 8.4, 2.1 Hz, 2H), 8.06 (d, J = 8.4 Hz, 2H), 8.33 (d, J = 2.1 Hz, 2H), 10.86 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 42.99, 115.61, 123.50, 127.87, 127.97, 133.63, 143.21, 165.08, 179.79, 181.67.

7.2.2. 2,7-Bis[2-(diethylamino)acetamido]anthraquinone (4)

Product **4** was obtained as yellow powder (yield 61%): mp 241 °C (EtOH). IR (KBr) (cm^{-1}) 1679, 1702, 3250. MS (EI, 70 eV) = 464 (M^+). ^{1}H NMR (300 MHz, DMSO): δ ppm 1.01 (t, J = 7.0 Hz, 12H), 2.61 (q, J = 6.9 Hz, 8H), 3.22 (s, 2H), 8.09 (d,J = 8.4 Hz, 2H), 8.13 (dd, J = 9.6, 1.2 Hz, 2H), 8.50 (s, 2H), 10.27 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 11.30, 47.17, 56.80, 115.73, 123.56, 127.57, 127.62, 133.58, 143.15, 170.41, 179.75, 181.80.

7.2.3. 2,7-Bis[2-(butylamino)acetamido]anthraquinone (5)

Product **5** was obtained as yellow powder (yield 40%): mp 125 °C (EtOH). IR (KBr) (cm^{-1}) 1671, 3331. MS (EI, 70 eV) = 464 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 0.94 (t, J = 7.2 Hz, 6H), 1.41 (m, 4H), 1.52 (m, 4H), 2.69 (t, J = 6.8 Hz, 4H), 3.41 (s, 4H), 8.09 (d, J = 1.5Hz, 2H), 8.25 (d, J = 8.4Hz, 2H), 8.31 (dd, J = 8.4, 2.1 Hz, 2H), 9.85 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 13.82, 20.23, 32.15, 50.04, 53.03, 116.29, 123.88, 128.96, 129.14, 34.53, 142.79, 170.73, 180.72, 182.45.

7.2.4. 2,7-Bis[2-(pyrrolidino)acetamido]anthraquinone (6)

Product **6** was obtained as pale yellow powder (yield 33%): mp 201 °C (EtOH). IR (KBr) (cm^{-1}) 1674, 1699, 3358. MS (EI, 70 eV) = 460.2 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.88 (m, *8*H), 2.71 (t, 8H), 3.32 (s, 4H), 8.09 (d, J = 2.1 Hz, 2H), 8.28 (d, J = 8.4Hz, 2H), 8.37 (dd, J = 8.7, 2.4 Hz, 2H), 9.58 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 24.11, 54.65, 59.79, 116.40, 124.07, 129.00, 129.17, 134.50, 142.88, 169.68, 180.71, 182.60.

7.2.5. 2,7-Bis[2-(piperidino)acetamido]anthraquinone (7)

Product **7** was obtained as yellow powder (yield 51%): mp 243 °C (EtOH). IR (KBr) (cm^{-1}) 1678, 1706, 3242. MS (EI, 70 eV) = 488 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.49 (m, 4H), 1.65 (m, 8H), 2.54 (t, J = 4.8 Hz, 8H), 3.09 (s, 4H), 8.07 (d, J = 2.1 Hz, 2H), 8.23 (d, J = 8.4 Hz, 2H), 8.28 (dd, J = 8.7, 1.8 Hz, 2H), 9.68 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 23.52, 26.20, 54.89, 62.77, 116.40, 124.11, 129.12, 129.25, 134.62, 142.87, 169.58, 180.83, 182.67.

7.2.6. 2,7-Bis[2-(dimethylamino)acetamido]anthraquinone (8)

Product **8** was obtained as yellow powder (yield 26%): mp 218 °C (EtOH). IR (KBr) (cm^{-1}) 1630, 1672, 1701, 3330. MS (EI, 70 eV) = 408 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.38 (s, 12H), 3.09 (s, 4H), 8.04 (d, J = 2.1 Hz, 2H), 8.19 (d, J = 8.4 Hz, 2H), 8.28 (dd, J = 8.4, 2.1 Hz, 2H), 9.55 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 45.98, 63.56, 116.39, 123.99, 129.05, 129.20, 134.54, 142.84, 169.32, 180.74, 182.50.

7.2.7. 2,7-Bis[2-(propylamino)acetamido]anthraquinone (9)

Product **9** was obtained as dark yellow powder (yield 30%): mp 143 °C (EtOH). IR (KBr) (cm^{-1}) 1670, 3333. MS (EI, 70 eV) = 436.2 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 0.98 (t, J = 7.5Hz, 6H), 1.56 (m, 4H), 2.66 (t, J = 6.9 Hz, 4H), 3.41 (s, 4H), 8.10 (d, J = 1.8 Hz, 2H), 8.25 (d, J = 8.1 Hz, 2H), 8.30 (dd, J = 8.4, 1.8 Hz, 2H), 9.84 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 11.17, 22.04, 50.51, 52.35, 115.45, 123.36, 127.62, 127.83, 133.71, 143.50, 171.08, 179.90, 181.98.

7.2.8. 2,7-Bis[2-(ethylamino)acetamido]anthraquinone (10)

Product **10** was obtained as dark brown powder (yield 55%): mp 145 °C (EtOH). IR (KBr) (cm^{-1}) 1670, 3330. MS (EI, 70 eV) = 408 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.18 (t, J = 7.2 Hz, 6H), 2.75 (q, J = 7.2 Hz, 4H), 3.42 (s, 4H), 8.12 (d, J = 1.8 Hz, 2H), 8.28

(d, J = 8.4 Hz, 2H), 8.35 (dd, J = 8.7, 2.1 Hz, 2H), 9.85 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 14.39, 42.78, 52.18, 115.50, 123.39, 127.62, 127.77, 133.69, 143.48, 171.07, 179.89, 181.96.

7.2.9. 2,7-Bis[2-(*N*-methylpiperazino)acetamido]anthraquinone (11)

Product **11** was obtained as yellow powder (yield 46%): mp 173 °C (EtOH). IR (KBr) (cm^{-1}) 1672, 1691, 1710, 3241. MS (EI, 70 eV) = 518 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.35 (s, 6H), 2.55 (s, 8H), 2.68 (s, 8H), 3.19 (s, 4H), 8.12 (d, J = 1.5 Hz, 2H), 8.28 (d, J = 8.7 Hz, 2H), 8.32 (dd, J = 8.7, 1.8 Hz, 2H), 9.55 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 45.27, 52.17, 54.02, 61.35, 115.83, 123.67, 127.77, 127.82, 133.73, 143.51, 169.05, 180.01, 182.06.

7.2.10. 2,7-Bis[2-(piperazino)acetamido]anthraquinone (12)

Product **12** was obtained as dark yellow powder (yield 19%): mp 173 °C (EtOH). IR (KBr) (cm^{-1}) 1643, 1668, 1699, 3332. MS (EI, 70 eV) = 490 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.60 (s, 8H), 3.00 (t, J = 4.8 Hz, 8H), 3.16 (s, 4H), 8.11 (d, J = 1.8 Hz, 2H), 8.28 (d, J = 8.7 Hz, 2H), 8.33 (dd, J = 8.7, 2.1 Hz, 2H), 9.58 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 44.81, 53.51, 62.07, 115.85, 123.71, 127.73, 127.77, 133.71, 143.43, 169.09, 179.98, 182.02.

7.2.11. 2,7-Bis[2-(isopropylamino)acetamido]anthraquinone (13)

Product **13** was obtained as brown powder (yield 44%): mp 158 °C (EtOH). IR (KBr) (cm^{-1}) 1672, 3323. MS (EI, 70 eV) = 436 (M$^+$). ^{1}H NMR (300 MHz, DMSO): δ ppm 1.01 (d, J = 6.0 Hz, 12H), 2.76 (m, 2H), 3.35 (s, 4H), 8.07 (dd, J = 8.4, 2.1 Hz, 2H), 8.14 (d, J = 8.7 Hz, 2H), 8.49 (d, J = 1.8 Hz, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 21.99, 47.65, 50.06, 115.45, 123.36, 127.64, 127.75, 133.70, 143.37, 171.20, 179.86, 181.93.

7.2.12. 2,7-Bis[2-(isobutylamino)acetamido]anthraquinone (14)

Product **14** was obtained as brown powder (yield 73%): mp 146 °C (EtOH). IR (KBr) (cm^{-1}) 1670, 3335. MS (EI, 70 eV) = 465 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 0.99 (d, J = 6.6Hz, 12H), 1.79 (m, 2H), 2.50 (d, J = 6.3 Hz, 4H), 3.41 (s, 4H), 8.13 (s, 2H), 8.28 (s, 4H), 9.86 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 20.06, 27.49, 52.60, 56.74, 115.38, 123.29, 127.61, 127.85, 133.71, 143.47, 171.13, 179.86, 181.95.

7.2.13. 2,7-Bis(3-chloropropionamido)anthraquinone (15)

Product **15** was obtained as yellow brown powder (yield 58%): mp 281 °C (EtOH). IR (KBr) (cm^{-1}) 1672, 1702, 3333. MS (EI, 70 eV) = 418.0 (M$^+$). ^{1}H NMR (300 MHz, DMSO): δ ppm 2.91 (t, J = 5.9 Hz, 4H), 3.90 (t, J = 5.9 Hz, 4H), 8.01 (d, J = 8.7 Hz, 2H), 8.09 (d, J = 8.7 Hz, 2H), 8.40 (s, 2H), 10.72 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 38.86, 39.82, 115.31, 123.14, 127.54, 127.67, 133.53, 143.44, 168.33, 179.63, 181.66.

7.2.14. 2,7-Bis[3-(diethylamino)propionamido]anthraquinone (16)

Product **16** was obtained as yellow powder (yield 55%): mp 193 °C (EtOH) (lit.[26] mp 215 °C). IR (KBr) (cm^{-1}) 1672, 1697, 3323. MS (EI, 70 eV) = 492.3 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.15 (t, J = 7.2 Hz, 12H), 2.54 (t, J = 5.7 Hz, 4H), 2.70 (q, J = 7.2 Hz, 8H), 2.79 (t, J = 5.6 Hz, 4H), 8.02 (s, 2H), 8.23 (s, 4H), 12.01 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 11.44, 33.27, 46.12, 48.79, 116.35, 124.37, 128.99, 129.11, 134.78, 144.04, 171.32, 181.06, 182.85.

7.2.15. 2,7-Bis[3-(butylamino)propionamido]anthraquinone (17)

Product **17** was obtained as yellow powder (yield 22%): mp 168 °C (EtOH). IR (KBr) (cm^{-1}) 1648, 1671, 3333. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.41 (s, 18H), 2.53 (t, J = 5.4 Hz, 4H), 2.67 (t, J = 5.4 Hz, 4H), 8.01 (d, J = 1.5 Hz, 2H), 8.23 (s, J = 3.0 Hz, 4H), 11.68 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 13.30, 19.38, 30.82, 34.37, 45.04, 46.43, 115.33, 123.23, 127.50, 127.83, 133.73, 143.38, 170.04, 179.36, 182.09.

7.2.16. 2,7-Bis[3-(pyrrolidino)propionamido]anthraquinone (18)

Product **18** was obtained as brown powder (yield 49%): mp 233 °C (EtOH) (lit.[26] mp 232 °C). IR (KBr) (cm^{-1}) 1645, 1672, 1698, 3331. MS (EI, 70 eV) = 488 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.95 (s, 8H), 2.57 (t, J = 6.0 Hz, 4H), 2.71 (s, 8H), 2.87 (t, J = 5.7 Hz, 4H), 7.98 (d, J = 2.1 Hz, 2H), 8.17 (dd, J = 8.4, 2.1 Hz, 2H), 8.24 (d, J = 8.7 Hz, 2H), 11.98 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 22.36, 35.73, 50.79, 57.83, 115.31, 123.15, 127.51, 127.87, 133.74, 143.37, 170.73, 179.93, 182.00.

7.2.17. 2,7-Bis[3-(piperidino)propionamido]anthraquinone (19)

Product **19** was obtained as yellowish brown powder (yield 34%): mp 266 °C (EtOH) (lit.[26] mp 240 °C). IR (KBr) (cm^{-1}) 1670, 1694, 3315. MS (EI, 70 eV) = 516 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.75 (t, J = 5.1 Hz, 12H), 2.53–2.65 (m, 12H), 2.69 (t, J = 4.8 Hz, 4H), 8.10 (d, J = 1.5 Hz, 2H), 8.19 (dd, J = 8.4, 2.4 Hz, 2H), 8.25 (d, J = 8.7 Hz, 2H), 12.05 (s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ ppm 24.09, 26.21, 32.44, 53.57, 54.03, 116.45, 124.34, 128.93, 129.14, 134.78, 144.23, 171.55, 181.28, 182.91.

7.2.18. 2,7-Bis[3-(dimethylamino)propionamido]anthraquinone (20)

Product **20** was obtained as yellowish powder (yield 20%): mp 196 °C (EtOH) (lit.[26] mp 202–203 °C). IR (KBr) (cm^{-1}) 1651, 1673, 1698, 3325. MS (EI, 70 eV) = 436 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.41 (s, 12H), 2.53 (t, J = 5.4 Hz, 4H), 2.67 (t, J = 5.4 Hz, 4H), 8.00 (s, 2H), 8.23 (s, 4H), 11.65 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 33.40, 44.38, 54.89, 116.87, 124.70, 129.08, 134.71, 144.04, 171.49, 179.99, 182.08.

7.2.19. 2,7-Bis[3-(propylamino)propionamido]anthraquinone (21)

Product **21** was obtained as yellow powder (yield 25%): mp 170 °C (EtOH). IR (KBr) (cm^{-1}) 1651, 1671, 1698, 3329. MS (EI, 70 eV) = 462 (M$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.41 (s, 14H), 2.54 (t, J = 6.0 Hz, 4H), 2.67 (t, J = 6.0 Hz, 4H), 8.01 (s, 2H), 8.23 (d, J = 1.8 Hz, 4H), 11.68 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 29.17, 29.57, 33.39, 44.37, 54.85, 116.87, 124.66, 129.04, 134.69, 144.04, 171.49, 181.22, 183.15.

7.2.20. 2,7-Bis[3-(ethylamino)propionamido]anthraquinone (22)

Product **22** was obtained as brown powder (yield 23%): mp 181 °C (EtOH). IR (KBr) (cm^{-1}) 1670, 3332. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.41 (s, 14H), 2.53 (t, J = 5.3 Hz, 4H), 2.67 (t, J = 5.3 Hz, 4H), 8.01 (s, 2H), 8.23 (s, 4H), 11.68 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 29.67, 33.38, 44.38, 54.88, 116.85, 124.67, 129.05, 134.68, 144.02, 171.47, 180.03, 182.07.

7.2.21. 2,7-Bis[3-(*N*-methylpiperazino)propionamido]anthraquinone (23)

Product **23** was obtained as yellow powder (yield 61%): mp 284 °C (EtOH). IR (KBr) (cm^{-1}) 1669, 1701, 3273. ^{1}H NMR (300 MHz, DMSO): δ ppm 2.40 (s, 6H), 2.57–2.76 (m, 24H), 8.15 (d, J = 9.6 Hz, 4H), 8.26 (d, J = 8.1 Hz, 2H), 11.69 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 23.51, 32.46, 45.35, 52.26, 55.32, 116.53, 124.38, 129.13, 129.21, 134.55, 144.10, 171.12, 181.29, 182.51.

7.2.22. 2,7-Bis[3-(piperazino)propionamido]anthraquinone (24)

Product **24** was obtained as yellow powder (yield 41%): mp 218 °C (EtOH). IR (KBr) (cm^{-1}) 1671, 1694, 3338. ^1H NMR (300 MHz, DMSO): δ ppm 2.33 (s, 8H), 2.54 (d, J = 5.7 Hz, 6H), 2.60 (d, J = 5.7 Hz, 4H), 2.67 (t, J = 4.5 Hz, 8H), 8.02 (dd, J = 8.4, 2.1 Hz, 2H), 8.13 (d, J = 8.4 Hz, 2H), 8.42 (d, J = 2.1 Hz, 2H), 10.75 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 33.60, 45.13, 53.37, 53.77, 115.36, 123.32, 127.61, 128.07, 133.91, 144.05, 171.03, 180.08, 182.23.

7.2.23. 2,7-Bis(4-chlorobutyramido)anthraquinone (25)

Product **25** was obtained as brown powder (yield 73%): mp 234 °C (EtOH). IR (KBr) (cm^{-1}) 1645, 1664, 1698, 3345. MS (EI, 70 eV) = 446.0 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 2.05 (m, 4H), 2.54 (t, J = 7.2 Hz, 4H), 3.71 (t, J = 6.5 Hz, 4H), 7.97 (dd, J = 8.7, 2.1 Hz, 2H), 8.05 (d, J = 8.7 Hz, 2H), 8.36 (d, J = 1.8 Hz, 2H), 10.54 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 21.18, 26.86, 67.72, 110.74, 119.04, 122.88, 128.34, 134.24, 150.95, 177.22, 178.72, 183.24.

7.2.24. 2,7-Bis(acetamido)anthraquinone (26)

Product **26** was obtained as yellowish brown powder (yield 42%): mp 340 °C (EtOH). IR (KBr) (cm^{-1}) 1645, 1670, 1691, 3333. MS (EI, 70 eV) = 322.1 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 2.21 (s, 6H), 8.00 (dd, J = 8.7, 2.1 Hz, 2H), 8.09 (d, J = 8.7 Hz, 2H), 8.38 (d, J = 2.1 Hz, 2H), 10.54 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 23.59, 115.02, 122.81, 127.21, 127.51, 133.42, 143.76, 168.56, 179.52, 181.67.

7.2.25. 2,7-Bis(propionamido)anthraquinone (27)

Product **27** was obtained as yellowish brown powder (yield 48%): mp 291 °C (EtOH). IR (KBr) (cm^{-1}) 1672, 1706, 3368. MS (EI, 70 eV) = 350.1 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 1.10 (t, J = 7.5 Hz, 6H), 2.39 (q, J = 7.5 Hz, 4H), 8.02 (dd, J = 8.7, 2.1 Hz, 2H), 8.10 (d, J = 8.7 Hz, 2H), 8.41 (d, J = 1.8 Hz, 2H), 10.46 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 8.72, 29.12, 115.12, 122.93, 127.20, 127.55, 133.50, 143.87, 172.26, 179.60, 181.77.

7.2.26. 2,7-Bis(butyramido)anthraquinone (28)

Product **28** was obtained as yellowish brown powder (yield 63%): mp 275 °C (EtOH). IR (KBr) (cm^{-1}) 1664, 1674, 1706, 3339. MS (EI, 70 eV) = 378.2 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 0.93 (t, J = 7.5 Hz, 6H), 1.63 (m, 4H), 2.36 (q, J = 7.2 Hz, 4H), 8.03 (dd, J = 8.4, 2.1 Hz, 2H), 8.10 (d, J = 8.4 Hz, 2H), 8.43 (d, J = 2.1 Hz, 2H), 10.48 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 12.97, 17.73, 37.86, 115.11, 122.87, 127.19, 127.48, 133.44, 143.77, 171.36, 179.50, 181.69.

7.2.27. 2,7-Bis(benzoamido)anthraquinone (29)

Product **29** was obtained as red brown powder (yield 30%): mp 224 °C (EtOH). IR (KBr) (cm^{-1}) 1678, 1703, 3320. MS (EI, 70 eV) = 446.1 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 7.57 (m, 6H), 8.00 (d, J = 8.1 Hz, 4H), 8.15 (d, J = 8.4 Hz, 2H), 8.30 (dd, J = 8.4, 1.8 Hz, 2H), 8.65 (d, J = 2.1 Hz, 2H), 10.80 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 116.69, 124.39, 127.44, 127.68, 127.81, 128.03, 131.66, 133.73, 144.15, 165.74, 180.12, 182.14.

7.2.28. 2,7-Bis(phenylacetamido)anthraquinone (30)

Product **30** was obtained as yellow powder (yield 74%): mp 224 °C (EtOH). IR (KBr) (cm^{-1}) 1644, 1668, 3322. MS (EI, 70 eV) = 474.2 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 3.72 (s, 4H), 7.30 (m, 10H), 8.05 (dd, J = 8.4, 2.1 Hz, 2H), 8.13 (d, J = 8.4 Hz, 2H), 8.44 (d, J = 2.1 Hz, 2H), 10.79 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 42.83, 115.54, 123.42, 126.26, 127.73, 128.74, 133.80, 134.95, 143.97, 169.72, 180.01, 182.08.

7.2.29. 2,7-Bis(phenylpropionamido)anthraquinone (31)

Product **31** was obtained as yellow powder (yield 45%): mp 268 °C (EtOH). IR (KBr) (cm^{-1}) 1672, 3336. MS (EI, 70 eV) = 502.1 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 2.71 (t, J = 7.8 Hz, 4H), 2.94 (t, J = 7.8 Hz, 4H), 7.23 (m, 10H), 8.02 (dd, J = 8.4, 2.1 Hz, 2H), 8.12 (d, J = 8.4 Hz, 2H), 8.43 (d, J = 2.1 Hz, 2H), 10.53 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 30.10, 41.17, 115.47, 123.41, 125.73, 127.68, 127.96, 128.07, 133.94, 140.65, 144.09, 171.22, 180.15, 182.30.

7.2.30. 2,7-Bis(cyclopropanecarbonamido)anthraquinone (32)

Product **32** was obtained as yellowish brown powder (yield 68%): mp 352 °C (EtOH). IR (KBr) (cm^{-1}) 1663, 3292. MS (EI, 70 eV) = 374.9 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 0.86 (d, J = 6.3 Hz, 8H), 1.83 (m, 2H), 8.02 (dd, J = 8.7, 2.1 Hz, 2H), 8.10 (d, J = 8.7 Hz, 2H), 8.42 (d, J = 2.1 Hz, 2H), 10.80 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 7.45, 14.37, 115.38, 123.25, 127.53, 128.05, 133.92, 144.11, 172.45, 180.09, 182.27.

7.2.31. 2,7-Bis(cyclopentanecarbonamido)anthraquinone (33)

Product **33** was obtained as yellowish brown powder (yield 51%): mp 281 °C (EtOH). IR (KBr) (cm^{-1}) 1647, 1674, 1702, 3342. MS (EI, 70 eV) = 430.9 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 1.54–1.89 (m, 16H), 2.83 (m, 2H), 8.04 (dd, J = 8.4, 2.1 Hz, 2H), 8.22 (d, J = 8.4 Hz, 2H), 8.46 (d, J = 2.1 Hz, 2H), 10.47 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 25.14, 29.45, 44.95, 115.42, 123.20, 127.39, 127.80, 133.70, 144.22, 174.95, 179.92, 182.12.

7.2.32. 2,7-Bis(cyclohexanecarbonamido)anthraquinone (34)

Product **34** was obtained as yellow powder (yield 42%): mp 269 °C (EtOH). IR (KBr) (cm^{-1}) 1670, 3323. MS (EI, 70 eV) = 458.9 (M^+). ^1H NMR (300 MHz, DMSO): δ ppm 1.16–1.46 (m, 12H), 1.63–1.84 (m, 8H), 2.37 (m, 2H), 8.02 (dd, J = 8.4, 1.8 Hz, 2H), 8.09 (d, J = 8.4 Hz, 2H), 8.44 (d, J = 1.8 Hz, 2H), 10.43 (s, 2H). ^{13}C NMR (300 MHz, DMSO): δ ppm 24.53, 24.76, 28.37, 44.42, 115.47, 123.29, 127.43, 127.76, 133.73, 144.26, 174.82, 179.97.

7.2.33. Cell culture and assessment of hTERT

Non-small lung cancer cells H1299[60] were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/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)[61] was grown in DMEM supplemented with 10% fetal calf serum, 100 U/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 transfected 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.

7.2.34. 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.[62,63] 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.

7.2.35. Telomere repeat amplification protocol (TRAP) assays

Telomerase activity was detected by a modified version of the TRAP protocol.[8,64,65] 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.

7.2.36. SEAP assay[66]

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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