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Synthesis, cytotoxicity and human telomerase inhibition activities of a series of 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione homologues

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ABSTRACT

A series of 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione tetracyclic analogues with different side chain were prepared using an various synthetic route via acylation, cyclization, condensation, and intramolecular heterocyclization. Tetracyclic system containing alkyl and aryl, aromatic and heterocyclic, linear and cyclic, polar and apolar, and basic and acids residues were incorporated. They were evaluated for their effects on telomerase activity, hTERT expression, cell proliferations, and in vitro cytotoxicity against NCI's 60 cell line human tumor screen. Compounds **4**, **11**, **12**, **14**, **15**, **16**, **17**, **19**, **20**, **23**, **25**, and **26** were selected by the NCI for one dose screening program and further studies on **4**, **23** and **25** where the curves cross these lines represent the interpolated values to cause 50% growth inhibition (GI_{50}), total growth inhibition (TGI) and 50% cell killing (LC_{50}), respectively. Further studies did not reveal any compound that showed potent and significant on telomerase inhibitory activity and hTERT repressing ability. Comparative testing of these compounds in the NCI's screen revealed varying levels of potency and differential cytotoxicity, apparently related to the unsaturation levels in and substitution patterns on the core ring system. It appeared that addition of a fourth planar aromatic system to a tricyclic chromophore might enhances potent cytotoxic agents, at a level equivalent to a second side chain in one of the tricyclic series. Although the exact mechanism of how this pharmacophore contributes to its activity is still unclear, however, the group in the extended arm of the tetracyclic system might contribute to proper binding to the residues within the grove of G-quadruplex structure.

1. Introduction

The mechanism of the cytotoxicity of anthracyclines is pleiotropic and its significance in cell growth inhibition seems to be highly specific and dependent on cell type and anthracycline drug.[1] The anthracycline antibiotics daunorubicin, and doxorubicin (Scheme 1) have been used widely as anticancer drugs for more than 30 years, but their cardiotoxicity limits their clinical use.[2] The clinical use of anthracyclines like doxorubicin and daunorubicin can be viewed as a sort of double-edged sword.[3] Doxorubicin and its analogues function as topoisomerase poisons through stabilization of the cleavable complex that forms as part of the catalytic cycle of the enzyme.[4] The detailed mechanism of how these compounds, all of which contain an anthraquinone core that forms between the DNA base pairs, the drug, and the enzyme in the minor groove has remained elusive. Anthracyclines and their anthraquinone derivatives remain 'evergreen' drugs with broad clinical indications but have an improvable pharmacological index. In an attempt to identify new molecular or pharmacophore targets for anticancer drugs, our attention has been focused on cytotoxicity and telomerase.

Telomerase activity is thought to be required for the development of cellular immortality and oncogenesis. Thus, considerable interest is focused on telomerase because of its potential uses in assays for cancer diagnosis, research into cell biology and for anti-telomerase drugs as a strategy for cancer chemotherapy.[5,6] At present, among different strategies pursued telomerase inhibitors in chemotherapy, the development of G-quadruplex stabilizers has emerged as a highly promising approach.[7,8] Herein, we report the design and synthesis of a series of non-nucleoside telomerase inhibitors, in an attempt to identify potent enzyme

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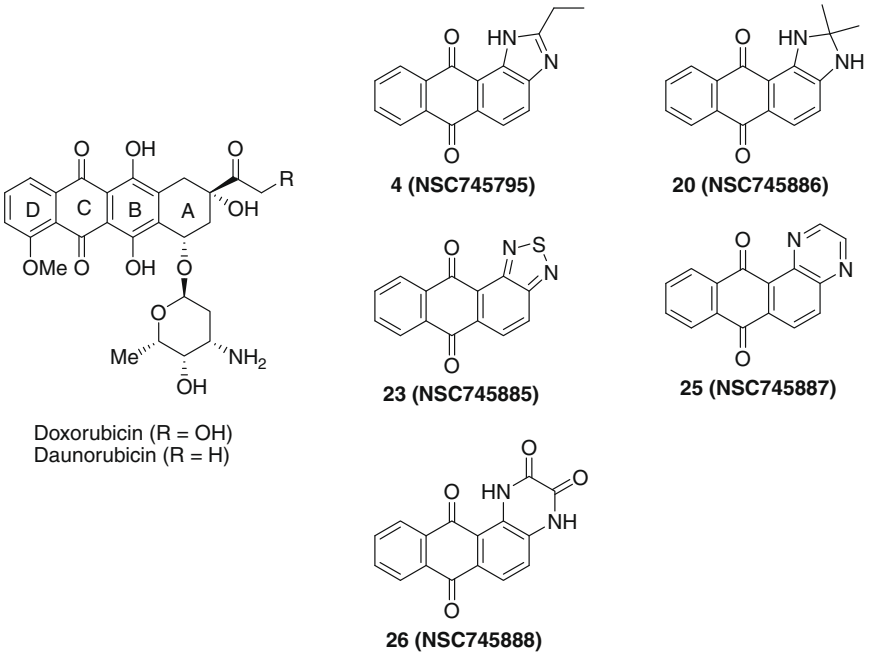


Scheme 1.

inhibitors and worked towards its structural modifications to develop some potential anti-cancer therapeutic leads compared to parent molecules.

As both benzimidazole and anthraquinone derivatives are known topoisomerase inhibitors and telomerase inhibitors,[9–13] the search for additional enzyme inhibitor is justified by the remarkable anticancer activity of imidazole-fused and heteroannelated anthraquinone derivatives and by the possibility that different enzyme poisons would exhibit different activity in cancer chemotherapy. Non-nucleoside (small-molecule) inhibitors that stabilize folded G-quadruplex structures might inhibit telomerase. Inhibitory agents from many chemical classes have been identified, many through screening, but their specificity of action is still in doubt. A specific inhibitor is expected to immediately inhibit activity but not cell division, produce telomerase shortening over multiple generations, and ultimately produce end-to-end chromosomal fusion and growth arrest.[14] In the course of our continuing search for new antitumor agents from anthraquinone moiety, we found that imidazole-fused and heteroannelated anthraquinone derivatives exhibited very similar and striking differential cytotoxicity profiles in the National Cancer Institute's (NCI) 60 cell line primary screen. Supposedly, an additional heterocyclic moiety or tetracyclic system might substantially increase the binding with their intracellular targets. We report here the synthesis methods, identification, and high cytotoxic activity of novel compounds for a variety of tumor cell lines. The synthesized compounds (**4**, **11**, **12**, **14**, **15**, **16**, **17**, **19**, **20**, **23**, **25**, **26**) were screened for in vitro cytotoxicity, besides; an attempt was made to understand their possible molecular mode of action and reportedly possesses diverse telomerase activity.

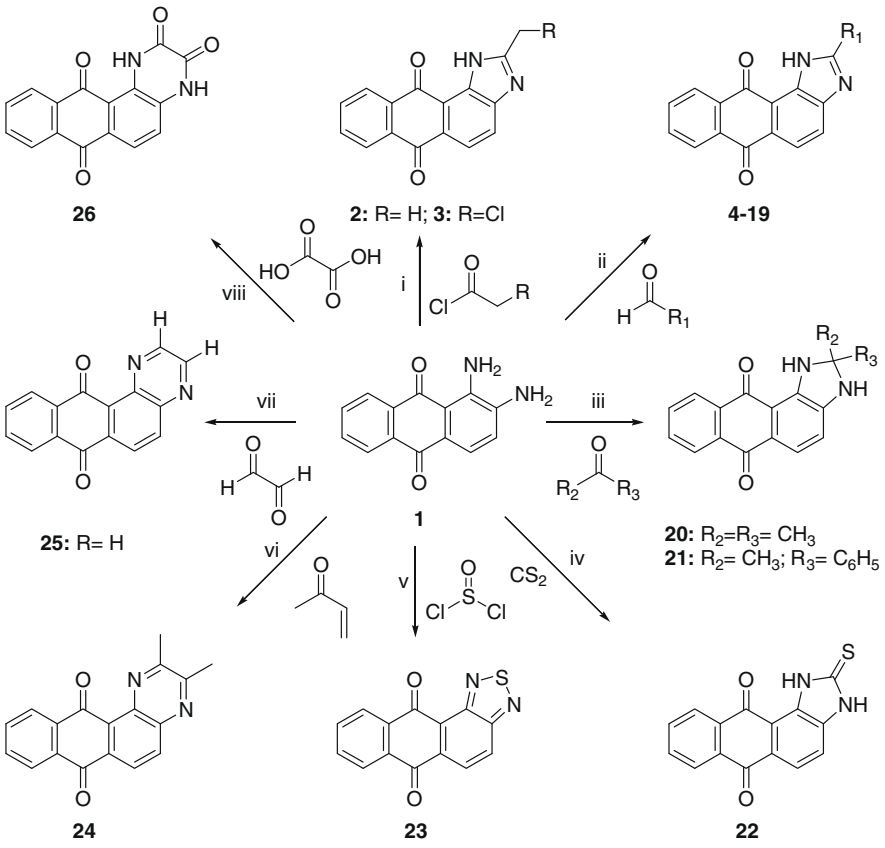
2. Results and discussion

2.1. Chemistry

The compounds were synthesized, starting from 1,2-diaminoanthraquinone. After considerable effort we were able to develop a process of fused tetracyclic system by which a series of 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione homologues can be synthesized and the preparation involved various synthetic route with appropriate yields (overall 23–89% in all steps) (Scheme 2): (i) acylation reaction of 1,2-diaminoanthraquinone **1** yielded the corresponding side chain compounds **2** and **3**; (ii) reaction of 1,2-diaminoanthraquinone **1** with various aldehydes and concd sulfuric acid yielded the corresponding side chain compounds **4–19**, respectively; (iii) reaction of 1,2-diaminoanthraquinone **1** with dry acetone and acetophenone and concd sulfuric acid yielded the corresponding side chain compounds **20** and **21**, respectively; (iv) reaction of 1,2-diaminoanthraquinone **1** with carbon disulfide and triethylamine yielded the corresponding compounds **22**; (v) reaction of 1,2-diaminoanthraquinone **1** with thionyl chloride and triethylamine yielded the corresponding compounds **23**; (vi) reaction of 1,2-diaminoanthraquinone **1** with methyl vinyl ketone and concd sulfuric acid yielded the corresponding compounds **24**; (vii) reaction of 1,2-diaminoanthraquinone **1** with glyoxal in ethanol yielded the corresponding compounds **25**; (viii) reaction of 1,2-diaminoanthraquinone **1** with oxalic acid and concd sulfuric acid yielded the corresponding compounds **26**. Compounds **4**–**19** were synthesized by direct one-step imidazole cyclization reaction using aldehydes to obtain various side chain substituted derivatives. 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 or chromatography. The molecular formulas of **2**–**26** were all determined by HRMS. The protons from thiadiazole and imidazole structure were also obvious from the ^{1}H NMR spectra.

2.2. Antiproliferative activity

The 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione derivatives were studies for antiproliferative activity against human cancer cell lines in NCI Drug Screen Program. Out of the synthesized derivatives, compounds **4**, **11**, **12**, **14**, **15**, **16**, **17**, **19**, **20**, **23**, **25**, and **26** have been selected by NCI as prototypes (Table 1). Those potential compounds were evaluated in the full panel of human tumor cell lines derived from nine cancer cell types



Scheme 2. Reagents and conditions: (i) **2**: DMF, 60 °C, 10 h; **3**: DMF, reflux, 10 h; (ii) R_1CHO, H_2SO_4 stir, rt; (**4**: $R_1 = C_2H_5$; **5**: $R_1 = CH(CH_3)_2$; **6**: $R_1 = (CH_2)_3CH_3$; **7**: $R_1 = CH(CH_3)C_2H_5$; **8**: $R_1 = C(CH_3)_3$; **9**: $R_1 = (CH_2)_6CH_3$; **10**: $R_1 = CH{=}CHC_2H_5$; **11**: $R_1 = C_6H_5$; **12**: $R_1 = C_6H_4N(CH_3)_2$ (*p*); **13**: $R_1 = C_6H_4NO_2$ (*p*); **14**: $R_1 = C_6H_3OH(p)(OCH_3)$ (*m*); **15**: $R_1 = C_6H_4(CH_3)$ (*p*); **16**: $R_1 = C_6H_4(Br)$ (*p*); **17**: $R_1 = C_6H_4(CN)$ (*p*); **18**: $R_1 = C_6H_3(OCH_3)_2$ (2,5); **19**: $R_1 = C_6H_3OCH_2O$ (iii) **20**: dry acetone, H_2SO_4 stir, rt, 48 h, $R_2 = R_3 = CH_3$; **21**: acetophenone, DMF, H_2SO_4 stir, rt, 72 h, $R_2 = CH_3$, $R_3 = C_6H_5$; (iv) CS_2, DMF, triethylamine, reflux, 10 h; (v) thionyl chloride, THF, triethylamine, stir, rt, 1 h; (vi) $CH_3COCH{=}CH_2$, DMF, H_2SO_4, stir, rt, 72 h; (vii) 40% glyoxal in EtOH, DMF, reflux, 16 h, Dean–Stark trap; (viii) oxalic acid, DMF, H_2SO_4, reflux, 16 h, Dean–Stark trap.

(leukemia, non-small cell lung cancer, colon cancer, CNS cancer, prostate cancer, melanoma, ovarian cancer, renal cancer and breast cancer). Each subpanel uses several cell lines and the dose–response curve is created by against the $\log_{10}$ of the corresponding drug concentration for each cell line by subpanel group. They were also 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. The growth percent values at 10^{-5} M for 60 cancer cell lines are shown in Table 1. For each of these parameters the averaged values of mean graph midpoint (MG_MID) were calculated. All of compounds, which reduced the growth of any one of the cell lines to 32% or less, were selected for further evaluation in the full panel of 60 human tumor cell lines. As shown in Table 2, three compounds (**4**, **23**, and **25**) of the 12 tested compounds showed significant cytotoxic activity have been selected for a 60-cell panel assay, along with GI_{50}, TGI and IC_{50}.

As a result, they were found that **4** (NSC745795), **23** (NSC745885) and **25** (NSC745887) have potent activity with GI_{50} conferred by 1.17–>100 μmol/L (11.5 μmol/L mean); 0.16–17.4 μmol/L (2.04 μmol/L mean); 0.07–29.3 μmol/L (5.24 μmol/L mean), respectively. Sensitive cell lines exhibit TGI and LC_{50} to **4** (NSC745795), all exhibited LC_{50} with >100 μmol/L and TGI with as little as 14.8 μmol/L; **23** (NSC745885), exhibited an LC_{50} with as little as 0.99 μmol/L and TGI with as little as 0.40 μmol/L; **25** (NSC745887), exhibited an LC_{50} with as little as 44.4 μmol/L and TGI with as little as 11.5 μmol/L, respectively. Results of the study extend the initial in vitro observation reported in the data above and confirm the importance of anticancer activity and telomerase inhibition.

The cancer panel, leukemia, melanoma and ovarian cancer lines were quite sensitive to compound **23**, while the non-small cell lung cancer lines, colon, CNS, renal, prostate and breast panels were less sensitive. It is surprising since compound **23** with heterocyclic thiadiazole tetracyclic-system has differences in cytotoxicity which might have a significant effect on their interaction with DNA. Compound **23** exhibited an GI_{50} as little as 0.16 μmol/L, LC_{50} with as little as 0.99 μmol/L and TGI with as little as 0.40 μmol/L for leukemia HL-60(TB).

It is especially notable that compound **4** with side chain $-CH_2CH_3$ displayed relatively potent and differential cytotoxic activity which had both cytotoxicity and telomerase activity whereas for cytocidal effects this difference was significant. However, it had significant influence on the spectrum of antiproliferative activity. Compounds **4** and **25** had also selective cytotoxicity for nine cancer cell types. It is especially notable that compound **25** have the best potent activity against leukemia K-562 with GI_{50} as little as 0.07 μmol/L, but LC_{50} and TGI with >100 μmol/L, respectively. The overall dose–response curves revealed that the leukemia subpanel was most sensitive for **23** and **25**, with GI_{50} of six leukemia cell lines ranging from 0.07 to 3.65 μmol/L. The ovarian cancer subpanel was ranked second most sensitive with GI_{50} between 0.55 and 29.3 μmol/L. Compounds **11**, **12**, **14**, **15**, **16**, **17**, **19**, **20** and **26** are lack of significant antiproliferative activity for cytotoxic activity. NCI 60 cell line panel assays from some selective compounds give only limited information and

Table 1
Cytotoxicity of selected compounds in the NCI in vitro 60-cell Drug Screen Program

Panel/cell line	Compounds/growth percent[a]											
	4 745795	**11** 745796	**12** 745794	**14** 745797	**15** 745798	**16** 745799	**17** 745883	**19** 745884	**23** 745885	**20** 745886	**25** 745887	**26** 745888
Non-small cell lung cancer												
A549/ATCC	68.24	78.97	113.27	97.86	55.73	94.98	105.67	101.67	104.18	94.62	47.13	106.10
EKVX	52.31	77.08	91.86	89.10	91.20	82.70	99.84	94.18	103.18	63.06	82.57	86.73
HOP-62	97.73	108.51	102.47	116.18	110.24	104.06	—	114.06	−100.00	—	—	—
HOP-92	41.43	50.68	89.88	120.18	77.27	83.53	56.20	65.33	—	5.10	—	−15.08
NCI-H226	75.94	80.48	89.30	100.41	100.62	102.60	103.05	97.05	97.80	89.68	86.72	95.83
NCI-H23	71.39	91.66	93.83	103.05	98.79	93.14	90.86	94.30	16.07	87.00	65.13	97.09
NCI-H322 M	31.42	77.44	94.79	107.02	84.56	167.19	126.73	149.65	125.82	79.66	96.35	159.05
NCI-H460	59.82	86.21	101.38	90.13	93.63	96.24	105.15	108.78	77.41	71.46	31.69	101.45
NCI-H522	41.48	80.36	87.08	71.47	84.67	76.50	141.18	83.27	—	38.90	24.09	100.37
Colon cancer												
COLO 205	69.65	92.05	208.28	100.71	107.64	109.96	112.74	126.21	111.54	90.57	71.56	101.14
HCC-2998	67.67	105.43	108.67	103.09	104.06	98.14	74.71	85.66	−0.74	136.96	−7.15	77.59
HCT-116	47.63	82.41	100.43	90.37	99.81	99.59	79.84	116.27	−50.00	54.72	53.27	91.14
HCT-15	42.18	86.34	106.53	102.65	100.32	96.58	100.63	105.78	1.48	64.64	52.15	99.34
HT29	64.46	83.16	104.64	101.15	105.54	98.76	86.41	95.79	116.95	103.48	81.90	114.87
KM12	72.94	75.53	97.95	113.20	106.39	101.82	98.58	97.78	98.60	51.55	61.00	97.82
SW-620	73.12	82.75	103.02	102.47	113.19	95.01	89.38	91.92	7.30	91.66	39.19	97.00
Breast cancer												
BT-549	—	—	—	—	94.14	—	—	—	—	—	—	—
HS 578T	−18.58	30.42	109.02	166.51	138.21	181.36	102.38	106.08	−7.40	68.42	76.75	79.00
MCF7	69.82	51.75	67.36	57.55	41.92	75.31	62.66	46.49	−50.74	83.58	39.68	85.17
MDA-MB-231/ATCC	78.01	81.43	94.44	87.94	97.59	92.27	87.12	107.23	−17.33	72.90	81.39	92.54
MDA-MB-435	84.39	100.59	105.81	102.71	105.10	109.92	113.02	109.72	−87.88	124.56	102.03	144.42
MDA-MB-468	—	—	—	—	—	—	94.95	96.41	−73.17	77.25	75.28	37.35
NCI/ADR-RES	74.44	90.19	108.06	102.96	103.93	93.74	101.88	104.74	−2.53	102.64	67.96	98.23
T-47D	63.05	65.06	119.31	83.92	75.98	97.28	85.09	106.53	−45.52	82.96	85.19	87.10
Ovarian cancer												
IGROV1	—	—	—	—	—	—	66.31	93.33	−88.34	−2.80	3.44	13.96
OVCAR-3	78.22	69.18	111.00	124.78	96.14	100.32	110.54	106.64	−15.47	78.22	71.65	93.18
OVCAR-4	48.81	73.43	98.31	83.02	96.83	94.06	101.11	100.52	−94.43	80.14	94.22	103.31
OVCAR-5	102.30	108.78	117.44	97.12	105.69	93.21	98.10	111.14	−20.07	96.48	106.59	110.43
OVCAR-8	72.94	91.00	111.08	93.42	96.39	96.34	98.86	96.47	−10.07	90.23	47.54	104.07
SK-OV-3	25.77	114.69	201.85	113.22	84.50	105.41	103.78	138.75	100.70	35.91	78.16	99.07
Leukemia												
CCRF-CEM	45.36	86.32	63.86	94.69	100.60	98.71	117.25	99.64	−30.81	74.01	—	90.48
HL-60(TB)	41.75	99.05	89.68	122.73	124.51	100.33	145.27	127.01	−21.77	128.84	102.34	125.76
K-562	64.43	76.04	96.09	95.49	102.37	108.81	54.91	80.62	49.61	101.39	37.23	105.90
MOLT-4	64.17	88.35	101.48	96.36	105.87	101.29	87.09	104.77	−40.09	116.41	22.91	121.52
RPMI-8226	56.17	61.11	78.39	81.02	91.52	95.18	113.71	96.21	−27.97	99.93	—	108.01
SR	52.55	95.53	118.40	73.92	113.66	92.44	84.08	91.74	4.96	72.44	82.92	108.60
Renal cancer												
786-0	76.60	111.09	109.24	109.05	112.66	111.34	124.28	107.42	−25.98	86.18	28.45	131.54
A498	28.88	76.95	86.10	121.53	175.54	117.25	96.21	91.71	130.09	80.60	98.83	99.78
ACHN	24.97	71.86	96.24	100.47	101.41	91.37	110.97	104.19	−94.31	50.17	46.52	81.33
CAK1-1	—	51.87	108.25	134.19	110.01	86.56	110.42	113.75	1.97	61.72	87.92	123.62
RXF 393	—	—	—	—	—	—	116.62	119.55	20.11	85.14	104.88	110.90
SN12C	68.53	87.49	94.51	102.48	103.28	111.51	104.85	101.03	−73.27	91.98	64.65	94.07
TK-10	33.82	81.97	115.78	174.50	153.53	280.47	180.05	—	96.99	62.39	126.89	274.15
UO-31	23.94	57.59	75.53	80.74	75.19	66.20	80.37	73.36	−79.30	30.65	51.45	61.43
Melanoma												
LOX IMVI	39.98	79.66	90.55	109.22	103.97	102.36	101.18	99.59	−50.63	61.88	44.18	96.94
M14	60.14	100.92	112.55	114.00	106.09	101.55	243.01	120.39	74.54	83.70	59.81	122.54
MALME-3 M	56.94	74.47	109.58	106.72	97.63	224.05	200.54	—	−71.54	155.47	180.88	149.83
SK-MEL-2	40.07	62.56	73.15	82.23	79.59	72.25	22.47	41.52	−73.16	8.04	6.66	21.96
SK-MEL-28	86.80	60.44	96.83	131.70	154.12	130.82	114.61	116.26	15.47	128.51	97.49	114.92
SK-MEL-5	60.75	65.18	93.80	88.06	87.97	87.08	102.76	98.73	8.52	86.45	66.57	98.99
UACC-257	39.69	—	103.22	—	—	—	104.79	116.58	−83.07	118.22	91.06	118.21
UACC-62	51.35	70.40	85.78	92.24	90.01	104.94	104.25	98.10	−82.32	67.58	90.47	95.25
Prostate cancer												
DU-145	61.60	84.27	99.89	88.12	98.85	112.86	97.74	106.77	40.76	76.05	93.32	102.68
PC-3	48.45	52.46	82.39	88.14	87.62	88.71	109.32	106.98	—	—	—	92.39
CNS cancer												
SF-268	80.37	91.93	95.43	106.83	106.35	106.47	99.17	100.23	16.29	90.16	67.98	98.26
SF-295	75.82	73.58	158.75	127.21	94.19	119.23	120.04	114.67	126.17	97.99	80.81	140.32
SF-539	83.16	96.17	111.18	101.80	91.69	102.86	103.92	107.60	−47.11	93.56	40.72	101.16
SNB-19	74.86	101.51	127.24	97.38	97.82	100.60	97.83	120.61	78.98	94.28	87.30	93.94
SNB-75	55.09	75.07	93.08	85.95	107.21	87.32	63.17	101.90	79.03	78.03	104.52	109.34

(*continued on next page*)

Table 1 (*continued*)

Panel/cell line	Compounds/growth percent[a]											
	4 745795	**11** 745796	**12** 745794	**14** 745797	**15** 745798	**16** 745799	**17** 745883	**19** 745884	**23** 745885	**20** 745886	**25** 745887	**26** 745888
U251	60.13	97.10	101.87	102.57	103.86	104.71	111.79	111.46	−89.26	87.84	63.47	95.92
Mean	58.42	80.85	103.68	102.39	100.84	106.31	103.78	102.18	2.68	80.41	69.36	100.83
Delta	77.00	50.43	39.82	44.84	58.92	40.11	81.31	60.66	102.68	83.21	76.51	115.91
Range	120.88	84.27	144.42	116.95	133.62	214.27	220.54	108.13	230.09	158.27	188.03	289.23

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

observations on mechanisms of drug action; for these DNA-affinic tetracyclic ring systems more precise physicochemical measurements are required which DNA polymorphic form is the target.

2.3. Telomerase activity

Binding of low-molecular weight ligands to quadruplexes was first studied using the classic DNA intercalator ethidium and the telomere sequence T_4G_4 from *Oxytricha*.[15] It is apparent that G-quadruplexes are a targets for drug action, with unique structural and electronic features, compared with the more established duplex or triplex DNA targets.[16] However, the G-quadruplex inhibitors studied have at least some affinity for duplex DNA, and thus, tend to exhibit conventional cytotoxicity, as well as particular telomerase-associated properties.[17] We evaluate the effects of these test derivatives on telomerase inhibition using PCR-based telomerase assay, and TRAP (telomeric repeat amplification protocol) assay. We also designed and expressed SEAP reporter gene under the control of hTERT promoter to monitor the expression of hTERT. Given the striking correlations between telomerase activity and antiproliferation capacity in tumor cells, we anticipated that analysis of tetracyclic chromophore telomerase might yield further insight into this relationship. We screened this series of derivatives, searching for compounds that exhibit inhibition of telomerase and/or SEAP assay with less cytotoxicity in MTT assay. Under the concentration of 100 μm, none of the synthesized compounds gave profound telomerase inhibitory activity (Fig. 1). Results with all test compounds showed MTT and SEAP inhibition activity in 1, 10 and 100 μM range are summarized in Table 3. We did not found any of the synthesized compounds showed specific repressive effects toward hTERT gene.

3. Conclusion

At present, we described a method of synthesizing 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione derivatives and comparing to their cytotoxicity and telomerase activity. A structure–activity relationship study was not conducted on a number of tetracyclic-system derivatives. In this investigation, we continue to focus our attention on the role of our systematic synthesized tetracyclic pharmacophore bearing the heterocycle and side chain linked to the planar anthraquinone moiety and to understand the basis of tetracyclic system selectivity. The physical, chemical and biological properties of anthraquinone-base derivatives are greatly affected by its various substituents of the planar ring system which considered as aglycon analogues 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 telomerase activity is regulated mostly at the transcriptional level for its catalytic subunit, *hTERT*, and partly at the post-translational level.[18] As shown in Table 3, 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 inhibit cell proliferation. Although the mechanism of preferential inhibition of compounds is still unclear to us, the compounds might have the potential to be developed into a drug for anti-cancer therapies.

To understand the unique antiproliferative activity pattern of test compounds in the NCI-60 cell line screen, we computed structure–activity relationships between the three compounds **4** (NSC745795), **23** (NSC745885) and **25** (NSC745887), respectively. They exhibited dose-dependent inhibition of proliferation in all 60 cancer cell lines. As shown in Tables 1 and 2, the cytotoxicity elicited by the three analogues upon human-derived carcinoma cells, is largely clustered within the leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer panels of the NCI 60 cell line screen. They may be appreciated from these representation of the data that the three analogues represent the most potent derivative from this series of tetracyclic derivatives. As shown in Table 2 for compound **4** (NSC 745795), the average concentration required to inhibit GI_{50} was 11.48 μmol/L with a range of 1.17 μmol/L (renal cancer: CAKI-1) to >100 μmol/L (leukemia: K-562; melanoma: SK-MEL-28; renal cancer: A498; breast cancer: HS 578T). As shown in Table 2 for compound **23** (NSC 745885), the average concentration required to inhibit GI_{50} was 2.04 μmol/L with a range of 0.16 μmol/L (leukemia cancer HL-60 TB) to 17.4 μmol/L (non-small cell lung cancer, NCI-H322 M). As shown in Table 2 for compound **25** (NSC745887), the average concentration required to inhibit GI_{50} was 5.24 μmol/L with a range of 0.07 μmol/L (leukemia, K562) to 29.3 μmol/L (ovarian cancer, OVCAR-5). In addition, >1 μmol/L (10^{-6} mol/L) was required to achieve LC_{50} in the majority of human tumor cell lines, cell line sensitive to compound **4** (NSC 745795), exhibited an LC_{50} with >1 μmol/L (10^{-6} mol/L) and TGI with as little as 14.8 μmol/L (ovarian cancer, IGROV1). Cell line sensitive to compound **23** (NSC 745885), exhibited an LC_{50} with as little as 0.99 μmol/L and also TGI with as little as 0.40 μmol/L (leukemia, HL-60 TB). Cell line sensitive to compound **25** (NSC 745887), exhibited an LC_{50} with as little as 44.4 μmol/L (melanoma, SK-MEL-5) and TGI with as little as 11.5 μmol/L (melanoma, HL-60 TB). Compounds **4** (NSC 745795), **23** (NSC 745885) and **25** (NSC 745887) exhibited dose-dependent inhibition of proliferation in all 60 cancer cell lines. Of the three, they were the most potent compounds during the all selected evaluation, with an average GI_{50} of 11.48 μmol/L for **4** (NSC 745795); with an average GI_{50} of 2.04 μmol/L for **23** (NSC 745885); with an average GI_{50} of 5.50 μmol/L for **25** (NSC 745887), respectively. All these data were summarized in Tables 1 and 2 and used for further analysis. Given the striking correlations between telomerase activity and proliferation capacity in tumor cells, we expected that analysis of tetracyclic analogues chromophore might yield further insight into designing better lead compounds for anti-cancer therapies. However, there is still no significant correlation between telomerase activity and cytotoxicity. The telomerase inhibitory data available

Table 2
In vitro anticancer activity by selected compounds **4** (NSC745795), **23** (NSC745885) and **25** (NSC745887) in the NCI's 60 human cancer cell lines

Panel/cell line (μM)	**4** (NSC 745795)			**23** (NSC 745885)			**25** (NSC 745887)		
	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Leukemia									
CCRF-CEM	16.5	>100	>100	1.08	4.1	>100	2.46	>100	>100
HL-60(TB)	2.72	64.1	>100	0.16	0.4	0.99	0.68	11.5	83.7
K-562	>100	>100	>100	1.92	15.9	41.2	0.07	>100	>100
MOLT-4	4.22	>100	>100	0.49	1.84	4.71	3.65	>100	>100
RPMI-8226	4.16	>100	>100	1.18	3.46	>100	1.61	>100	>100
SR	6.71	>100	>100	—	—	—	—	—	—
Non-small cell lung cancer									
A549/ATCC	28.5	>100	>100	14.7	36.6	91	4.53	>100	>100
EKVX	4.6	>100	>100	1.55	3.45	7.66	3.45	72.1	>100
HOP-62	32.2	>100	>100	1.28	3.31	8.53	5.64	>100	>100
HOP-92	9.84	7.58	>100	2.52	6.64	>100	22.5	>100	>100
NCI-H226	2.46	>100	>100	4.48	21.6	63.7	16.6	95.2	>100
NCI-H23	12.1	>100	>100	1.44	6.04	>100	12	70.3	>100
NCI-H322M	4.87	>100	>100	17.4	47.8	>100	16.3	96.1	>100
NCI-460	5.27	>100	>100	3.27	14.3	47.3	2.01	26.9	>100
NCI-H522	28.3	>100	>100	1.66	3.93	9.31	4.64	32.5	>100
Colon cancer									
COLO 205	8.95	>100	>100	13.4	28.9	62.4	3.4	28.4	>100
HCC-2998	18.8	>100	>100	16.4	33.1	66.6	1.4	83.5	>100
HCT-116	5.9	>100	>100	1.93	5.4	>100	12.3	>100	>100
HCT-15	3.39	>100	>100	1.71	3.72	8.12	4.63	84.3	>100
HT29	16.1	>100	>100	13.4	50.4	>100	7.01	>100	>100
KM12	7.69	>100	>100	1.96	5.54	59.7	5.26	49.5	>100
SW-620	19.8	>100	>100	1.79	3.5	6.84	1.71	30.8	>100
CNS cancer									
SF-268	20.3	>1000	>100	2.17	5.77	27.7	4.98	>100	>100
SF-295	4.15	>100	>100	1.9	4.04	8.56	4.35	32.3	>100
SF-539	11.7	>100	>100	1.46	2.94	5.91	2.18	53.7	>100
SNB-19	18.1	>100	>100	2.28	5.82	26	13.4	>100	>100
SNB-75	62.5	>100	>100	2.03	5.2	18.6	11.9	>100	>100
U251	16.3	>100	>100	1.61	2.96	5.44	5.38	42.9	>100
Melanoma									
LOX IMV1	4.56	>100	>100	0.82	2.13	4.86	4.2	38.9	>100
MALME-3M	8.55	>100	>100	1.66	3.15	5.96	4.48	20.7	70.8
M14	7.42	>100	>100	2.15	4.85	>100	5.14	>100	>100
MDA-MB-435	30.3	>100	>100	1.91	3.31	5.76	11.1	23.7	50.4
SK-MEL-2	—	—	—	1.68	4.22	22.5	12.7	55.9	>100
SK-MEL-28	>100	>100	>100	1.85	3.38	6.18	21.0	91.1	>100
SK-MEL-5	12.7	59.1	>100	1.58	2.92	5.41	3.93	17.1	44.4
UACC-257	47.2	>100	>100	1.74	3.51	7.09	13	68.3	>100
UACC-62	8.09	>100	>100	1.45	2.9	5.79	13.3	56.1	>100
Ovarian cancer									
IGROV1	1.54	14.8	>100	1.36	2.94	6.35	2.55	35.9	>100
OVCAR-3	16.5	>100	>100	0.55	2.46	11.1	2.34	12.5	>100
OVCAR-4	25.7	>100	>100	0.65	2.17	5.57	10.5	>100	>100
OVCAR-5	77.4	>100	>100	1.91	4.11	8.84	29.3	>100	>100
OVCAR-8	23.2	>100	>100	1.66	4.68	59.8	5.07	>100	>100
NCI/ADR-RES	18.9	>100	>100	1.86	12.6	>100	5.68	>100	>100
SK-OV-3	2.59	>100	>100	7.71	22.5	53.4	6.95	>100	>100
Renal cancer									
786-0	43.6	>100	>100	6.17	19.3	50.4	8.87	>100	>100
A498	>100	>100	>100	12.4	25.4	52	9.31	50.3	>100
ACHN	3.29	>100	>100	1.46	2.77	5.27	3.85	>100	>100
CAKI-1	1.17	>100	>100	1.61	2.96	5.44	5.83	>100	>100
RXF 393	6.95	>100	>100	1.75	4.4	14.6	6.43	>100	>100
SN12C	13.6	>100	>100	1.33	3.17	7.56	13.2	92.7	>100
TK-10	12.1	>100	>100	2.85	10.9	50.4	5	>100	>100
UO-31	1.33	>100	>100	1.62	3.12	6.01	3.02	85.9	>100
Prostate cancer									
PC-3	28.2	>100	>100	3.23	>100	>100	—	>100	>100
DU-145	9.43	>100	>100	1.67	3.24	6.27	11.4	34.3	>100
Breast cancer									
MCF7	13	>100	>100	1.53	3.34	7.29	0.72	29.8	>100
MDA-MB-231/ATCC	8.86	>100	>100	1.75	5.9	>100	19.1	>100	>100
HS 578T	>100	>100	>100	1.41	5.02	56	14.1	91.4	>100
BT-549	9.3	>100	>100	1.63	3.13	6.01	12.2	93.5	>100
T-47D	3.3	>100	>100	1.39	3.39	8.27	12.6	75.1	>100
MDA-MB-468	8.29	>100	>100	—	—	—	—	—	—

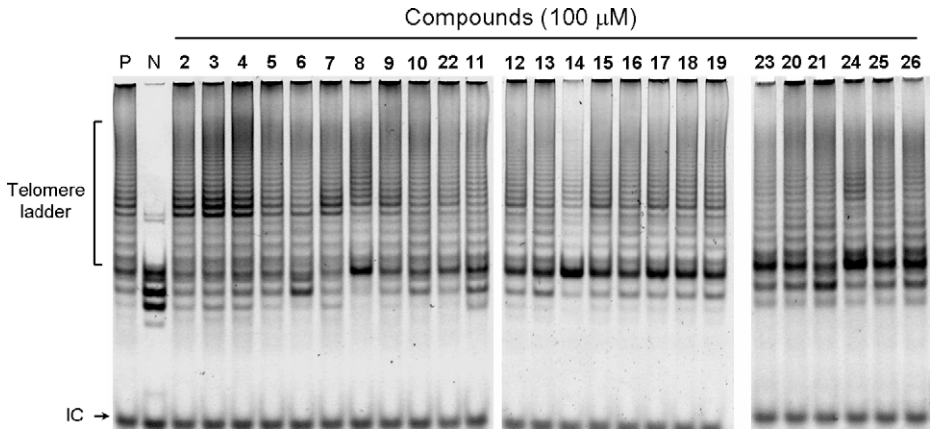


Figure 1. Inhibition of telomerase activity by 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione homologues which tested concentration was 100 μM. [P—positive control (no inhibitor); N—negative control (RNase A-treated cell extract, no inhibitor)].

here for tetracyclic anthraquinone system have demonstrated that activity at the micromolar level can be achieved with pharmacologically acceptable compounds. Their telomerase selectivity is relatively modest, and this, combined with cytotoxicity at comparable levels, suggests that these types of tetracyclic compounds may produce potential candidates suitable for in vivo preclinical evaluation.

4. Experimental

Melting points were determined with a Büchi B-545 melting point apparatus and are uncorrected. All reactions were monitored by TLC (Silica Gel 60 F_{254}). ^{1}H NMR: Varian GEMINI-300 (300 MHz) and Brucker AM-500 (500 MHz); δ values are in ppm relative to TMS as an internal standard. Fourier-transform IR spectra (KBr): Perkin–Elmer 983G spectrometer. Mass spectra (EI, 70 eV, unless otherwise stated): Finnigan MAT TSQ-46, Finnigan MAT TSQ-700 (Universität Regensburg, Germany) and Finnigan MAT LCQ-MS (National Research Institute of Chinese Medicine, Taipei, Taiwan). Typical experiments illustrating the general procedures for the preparation of the anthraquinones are described below.

4.1. 2-Methyl-1(3)*H*-anthrasimidazole-6,11-dione (2)

1,2-Diaminoanthraquinone (1.19 g, 5 mmol) and acetyl chloride (0.5 mL, 6 mmol) were dissolved in DMF (30 mL) and stirred at 130 °C for 10 h. Once the starting materials were consumed, as indicated by TLC, the reaction mixture was cooled to room temperature. Water (200 mL) was added to precipitate out the crude product. The resulting precipitate was collected by filtration, washed with ethanol and purified by crystallization from ethanol to afford desired compounds (R_f = 0.79 at ethyl acetate/dichlorometh-

Table 3
Effects of 1,2-heteroannelated anthraquinones and anthra[1,2-*d*]imidazole-6,11-dione homologues on activating or repressing hTERT expression and telomerase activity

Compound	Cell type (H1299) (inhibition μM ± SD)					
	MTT			SEAP		
	1 (μM)	10 (μM)	100 (μM)	1 (μM)	10 (μM)	100 (μM)
2	64 ± 7	44 ± 8	33 ± 6	101 ± 3	82 ± 5	19 ± 12
3	80 ± 8	79 ± 7	26 ± 6	94 ± 4	81 ± 5	5 ± 7
4	88 ± 7	82 ± 9	37 ± 4	70 ± 7	59 ± 1	25 ± 12
5	95 ± 5	73 ± 15	34 ± 24	83 ± 5	76 ± 8	13 ± 8
6	85 ± 8	70 ± 8	20 ± 9	91 ± 4	84 ± 3	36 ± 9
7	77 ± 9	83 ± 2	43 ± 5	67 ± 9	58 ± 12	11 ± 14
8	83 ± 5	81 ± 7	56 ± 7	88 ± 4	87 ± 6	29 ± 7
9	85 ± 6	69 ± 5	40 ± 5	89 ± 5	88 ± 3	32 ± 6
10	69 ± 5	48 ± 4	14 ± 5	63 ± 1	34 ± 1	13 ± 5
11	79 ± 11	72 ± 10	34 ± 4	81 ± 6	78 ± 8	10 ± 7
12	88 ± 10	62 ± 12	22 ± 16	92 ± 5	73 ± 10	21 ± 10
13	84 ± 9	52 ± 4	33 ± 9	85 ± 7	62 ± 5	6 ± 7
14	71 ± 9	70 ± 9	38 ± 11	85 ± 6	50 ± 8	8 ± 6
15	75 ± 9	72 ± 13	16 ± 15	86 ± 6	82 ± 2	29 ± 6
16	96 ± 9	85 ± 8	20 ± 17	89 ± 4	71 ± 9	36 ± 4
17	80 ± 2	77 ± 10	19 ± 11	91 ± 5	83 ± 9	30 ± 7
18	91 ± 12	39 ± 1	13 ± 3	77 ± 8	76 ± 4	41 ± 4
19	79 ± 4	67 ± 9	40 ± 4	61 ± 4	61 ± 4	34 ± 6
20	98 ± 6	93 ± 3	31 ± 5	96 ± 8	73 ± 7	9 ± 4
21	99 ± 2	99 ± 6	29 ± 9	99 ± 2	97 ± 1	29 ± 6
22	101 ± 4	99 ± 2	54 ± 5	97 ± 4	100 ± 5	31 ± 8
23	40 ± 7	16 ± 1	17 ± 7	89 ± 9	10 ± 13	9 ± 13
24	78 ± 3	69 ± 8	25 ± 14	95 ± 6	88 ± 8	26 ± 10
25	98 ± 4	13 ± 9	6 ± 4	105 ± 10	26 ± 8	24 ± 7
26	85 ± 5	65 ± 9	25 ± 14	101 ± 4	89 ± 8	20 ± 8

ane = 1:4). The solid material was isolated in 67% yield. Mp >400 °C (EtOH).[17] FT-IR (KBr; ν cm^{-1}) 1667 (CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 2.72 (s, 3H), 7.75–7.82 (m, 2H), 7.93 (d, J = 8.4 Hz, 1H), 8.13 (d, J = 8.4 Hz, 1H), 8.19–8.23 (m, 1H), 11.01 (br, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 23.89, 120.23, 121.22, 125.29, 126.19, 126.75, 127.19, 128.17, 128.87, 132.98, 134.18, 134.42, 148.22, 158.09, 182.43 (*C*O), 185.13 (*C*O). EI-MS m/z: 262 (M$^+$, 100).

4.2. 2-Chloroacetyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (3)

1,2-Diaminoanthraquinone (1.19 g, 5 mmol) and chloroacetyl chloride (0.5 mL, 6 mmol) were dissolved in DMF (30 mL) and stirred at 50–60 °C for 10 h. Water (200 mL) was added to precipitate out the crude product. The resulting precipitate was collected by filtration, washed with ethanol and purified by crystallization from ethanol to afford desired compounds (R_f = 0.5 at ethyl acetate/dichloromethane = 1:4). The solid material was isolated in 80% yield. Mp 272–273 °C (EtOH).[19] FT-IR (KBr; ν cm^{-1}) 3359 (NH), 1660 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 4.92 (s, 2H), 7.80–7.83 (m, 2H), 8.08 (d, J = 8.4 Hz, 1H), 8.24 (d, J = 8.4 Hz, 1H), 8.26–8.35 (m, 2H), 11.21 (br, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 37.80, 119.35, 121.27, 125.95, 126.83, 127.40, 129.06, 132.35, 133.47, 133.64, 134.88, 135.10, 148.89, 156.93, 183.04 (*C*O), 183.83 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 297.0425; found, 297.0426.

4.3. General procedure for the synthesis of compounds (4–19)

Various aldehyde (5 mmol) was added dropwise to a solution of 1,2-diaminoanthraquinone (1.19 g, 5 mmol) and concd sulfuric acid (0.1 mL) in DMF (30 mL). The reaction mixture was stirred for 1 h at room temperature. The mixture was treated with crushed ice and the product was extracted into dichloromethane. After drying the organic layer over anhydrous Na_2SO_4, solvent was removed and the residue was purified by crystallization from EtOH to afford desired compounds.

4.3.1. 2-Ethyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (4)

Product **4** was obtained as brown powder (yield 39%): mp 193–195 °C (EtOH) (R_f = 0.75 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 1669 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.51 (t, J = 7.5 Hz, 3H), 3.05 (q, J = 7.5 Hz, 2H), 7.73–7.81 (m, 2H), 7.99 (d, J = 8.4 Hz, 1H), 8.16 (d, J = 8.4 Hz, 1H), 8.21–8.23 (m, 1H), 8.27–8.31 (m, 1H), 10.85 (br, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 11.87, 22.89, 117.74, 121.50, 125.21, 126.47, 127.55, 128.21, 132.72, 133.24, 133.72, 133.99, 134.37, 148.90, 161.64, 182.81 (*C*O), 185.15 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 277.0971; found, 277.0975; calcd for $C_{17}H_{12}N_2O_2Na^+$ [M+Na]$^+$: 299.0971; found: 299.0794.

4.3.2. 2-Isopropyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (5)

Product **5** was obtained as yellow powder (yield 41%): mp 117–119 °C (EtOH) (R_f = 0.7 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3445 (NH), 1662 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.56 (d, J = 6.6 Hz, 6H), 3.40 (s, J = 6.6 Hz, 1H), 7.78–7.85 (m, 2H), 8.11 (d, J = 8.4 Hz, 1H), 8.23 (d, J = 8.4 Hz, 1H), 8.25–8.36 (m, 2H), 10.88 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 21.15, 29.21, 117.66, 121.36, 125.21, 126.32, 127.42, 128.05, 132.49, 133.10, 133.61, 133.86, 134.24, 148.71, 165.35, 181.05 (*C*O), 182.73 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 291.1120; found, 291.1123.

4.3.3. 2-Butyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (6)

Product **6** was obtained as brown powder (yield 36%): mp 192–193 °C (EtOH) (R_f = 0.65 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 1669 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.00 (t, J = 7.2 Hz, 3H), 1.50 (sx, J = 7.5 Hz, 2H), 1.93 (qt, J = 7.8 Hz, 2H), 3.04 (t, J = 7.5 Hz, 2H), 7.62–7.83 (m, 2H), 8.03 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.1 Hz, 1H), 8.24–8.35 (m, 2H), 10.83 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 12.98, 21.78, 28.60, 29.27, 117.32, 121.07, 124.64, 125.98, 127.08, 127.83, 132.17, 132.84, 133.20, 133.61, 133.86, 148.25, 160.29, 182.31 (*C*O), 184.78 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 305.1276; found, 305.1282. Calcd, [M−H]$^-$: 303.1131; found, 303.1135.

4.3.4. 2-*sec*-Butyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (7)

Product **7** was obtained as yellow powder (yield 40%): mp 117–119 °C (EtOH) (R_f = 0.57 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 1665 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.00 (t, J = 7.2 Hz, 3H), 1.52 (d, J = 6.9 Hz, 3H), 1.82–2.02 (m, 2H), 3.04 (sx, J = 7.2 Hz, 1H), 7.62–7.83 (m, 2H), 8.03 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.1 Hz, 1H), 8.24–8.35 (m, 2H), 10.83 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 11.09, 18.09, 28.40, 35.71, 117.39, 121.07, 124.75, 125.95, 127.09, 127.84, 131.92, 132.83, 133.22, 133.59, 133.87, 148.06, 164.30, 182.31 (*C*O), 184.82 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 305.1276; found, 305.1280. Calcd, [M−H]$^-$: 303.1131; found, 303.1137.

4.3.5. 2-*tert*-Butyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (8)

Product **8** was obtained as yellow powder (yield 37%): mp 209–210 °C (EtOH) (R_f = 0.80 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3568 (NH), 1664 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.58 (s, 9H), 7.77–7.84 (m, 2H), 8.08 (d, J = 8.4 Hz, 1H), 8.21 (d, J = 8.4 Hz, 1H), 8.25–8.28 (m, 1H), 8.33–8.36 (m, 1H), 10.83 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 29.24, 117.79, 121.47, 125.41, 126.39, 127.56, 128.17, 132.70, 133.23, 133.74, 133.96, 134.37, 148.73, 168.00, 182.77 (*C*O), 185.26 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 305.1276; found, 305.1283. Calcd, [M−H]$^-$: 303.1131; found, 303.1136.

4.3.6. 2-Heptyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (9)

Product **9** was obtained as brown powder (yield 38%): mp 85–87 °C (EtOH) (R_f = 0.85 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3447 (NH), 1664 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 0.87–0.91 (m, 3H), 1.26–1.35 (m, 6H), 1.56 (sx, J = 7.0 Hz, 2H), 2.36 (q, J = 7.0 Hz, 2H), 2.71 (t, J = 7.0 Hz, 2H), 7.75–7.81 (m, 2H), 8.04 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 8.0 Hz, 1H), 8.23–8.25 (m, 1H), 8.31–8.33 (m, 1H), 10.93 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 14.08, 22.63, 27.99, 28.79, 29.24, 29.46, 31.79, 117.49, 121.66, 125.28, 126.37, 127.54, 130.56, 133.27, 133.67, 134.06, 134.31, 137.37, 149.40, 158.89, 182.69 (*C*O), 185.25 (*C*O). HR-MS (ESI-TOF) m/z calcd, [M+H]$^+$ 347.1754; found, 347.1752.

4.3.7. (*E*)-2-(But-1-enyl)-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (10)

Product **10** was obtained as brown powder (yield 33%): mp 117–119 °C (EtOH) (R_f = 0.57 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 1664 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 0.98 (t, J = 6.9 Hz, 3H), 1.94–1.98 (m, 2H), 6.16–6.29 (m, 1H), 6.51 (d, J = 18 Hz, 1H), 7.68 (d, J = 8.4 Hz, 1H), 7.82–7.89 (m, 2H), 8.14 (d, J = 8.1 Hz, 1H), 8.27–8.35 (m, 2H), 10.74 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 14.39, 27.40, 117.37, 120.03, 121.07, 124.75, 125.95, 127.09, 127.84, 131.92, 132.83, 133.22, 133.59, 133.87, 134.90, 135.37, 149.06, 18 2.73 (*C*O), 185.18 (*C*O). EI-MS m/z 302 (M$^+$, 100).

4.3.8. 2-Phenyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (11)

Product **11** was obtained as yellowish brown powder (yield 74%): mp 232–233 °C (EtOH) (R_f = 0.55 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3296 (NH),1660 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.57 (t, J = 3 Hz, 3H), 7.89 (m, 2H),

8.03 (d, J = 8.4 Hz, 1H), 8.08 (d, J = 8.4 Hz, 1H), 8.16 (m, 2H), 8.40 (dd, J = 6.3 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 119.62, 121.72, 125.06, 126.85, 127.42, 128.79, 128.86, 129.41, 129.50, 131.72, 133.72, 133.77, 134.92, 135.07, 149.26, 158.25, 183.06 (*C*O), 183.79 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 325.0971; found, 325.0973. EI-MS m/z 324 (M^+, 100), 325 (19).

4.3.9. 2-(4-*N*,*N*-Dimethylamino)phenyl-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (12)

Product **12** was obtained as dark brown powder (yield 79%): mp 239–240 °C (EtOH) (R_f = 0.60 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3404 (NH),1659 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 3.09 (s, 6H), 6.81 (d, 2H), 7.79–7.82 (m, 3H), 8.03–8.22 (m, 3H), 8.27–8.36 (m, 2H), 11.10 (br, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 39.95, 111.65, 115.23, 117.13, 121.83, 124.24, 126.33, 127.37, 127.44, 128.27, 133.27, 133.45, 133.54, 134.12, 150.11, 152.10, 157.59, 182.47 (*C*O), 185.09 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 368.1393; found, 368.1393. EI-MS m/z 366 (27), 367 (M^+, 100), 368 (20).

4.3.10. 2-(4-Nitrophenyl)-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (13)

Product **13** was obtained as red brown powder (yield 89%): mp 342–343 °C (EtOH) (R_f = 0.60 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3460 (NH),1657 (CO), 1517, 1345 (NO_2). ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.79–7.82 (m, 3H), 7.14 (d, J = 8.1 Hz, 1H), 8.23 (d, J = 8.1 Hz, 1H), 8.23–8.32 (m, 2H), 8.39 (d, J = 8.1 Hz, 2H), 8.58 (d, J = 8.1 Hz, 2H), 10.15 (br, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 117.81, 122.43, 123.62, 125.24, 125.88, 126.10, 127.92, 133.22, 133.36, 134.53, 143.08, 146.39, 146.77, 155.89, 172.18, 178.35, 179.40, 183.20 (*C*O), 185.56 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 370.0822; found, 370.0823. EI-MS m/z 249 (100), 369 (M^+, 35).

4.3.11. 2-(4-Hydroxy-3-methoxyphenyl)-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (14)

Product **14** was obtained as brown powder (yield 47%): mp 230–231 °C (EtOH) (R_f = 0.20 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3411 (NH), 1664 (CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 3.91 (s, 3H), 6.90 (d, J = 8.4 Hz, 1H), 7.81–7.88 (m, 3H), 7.92–7.96 (m, 3H), 7.99 (s, 1H), 8.11 (d, J = 8.4 Hz, 2H), 9.78 (br, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 56.57, 112.72, 116.37, 119.21, 119.65, 122.05, 122.95, 123.88, 126.81, 127.41, 128.42, 133.50, 133.64, 134.87, 135.09, 148.48, 150.87, 158.33, 182.85 (*C*O), 183.79 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 370.1026; found, 370.1025. EI-MS m/z 369 (57), 370 (M^+, 100).

4.3.12. 2-*p*-Tolyl-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (15)

Product **15** was obtained as yellowish brown powder (yield 76%): mp 256–257 °C (EtOH) (R_f = 0.65 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3397 (NH), 1659 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.46 (s, 3H), 7.37 (d, J = 8.1 Hz, 2H), 7.79 (t, J = 3.6 Hz, 2H), 8.03 (d, J = 7.8 Hz, 2H), 8.08 (d, J = 8.4 Hz, 1H), 8.21 (d, J = 8.4 Hz, 1H), 8.24–8.34 (m, 2H), 11.21 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 21.58, 117.89, 121.96, 125.44, 125.75, 126.46, 127.00, 127.58, 128.43, 130.00, 133.20, 133.26, 133.72, 133.99, 134.38, 142.05, 149.50, 156.86, 182.60 (*C*O), 185.16 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 339.1128; found, 339.1128. EI-MS m/z 338 (M^+, 100), 339 (24).

4.3.13. 2-(4-Bromophenyl)-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (16)

Product **16** was obtained as red brown powder (yield 75%): mp 302–303 °C (EtOH) (R_f = 0.40 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3391 (NH), 1658 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.72 (d, J = 8.7 Hz, 2H), 7.80–7.83 (m, 2H), 8.06 (d, J = 8.7 Hz, 2H), 8.13 (d, J = 8.4 Hz, 1H), 8.25 (d, J = 8.4 Hz, 1H), 8.27–8.36 (m, 2H), 11.29 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 122.18, 125.86, 126.11, 126.57, 127.64, 127.69, 128.50, 128.89, 132.61, 133.20, 133.35, 133.87, 134.01, 134.57, 149.40, 155.62, 182.63 (*C*O), 185.25 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 403.0085; found, 403.0073. Calcd, $[M-H]^-$ 400.9939; found, 400.9923. EI-MS m/z 402 (M^+, 100), 404 (97).

4.3.14. 2-(4-Cyanophenyl)-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (17)

Product **17** was obtained as yellowish brown powder (yield 77%): mp 353–354 °C (EtOH) (R_f = 0.65 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3341 (NH), 2229 (CN), 1667 (CO). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.80–7.85 (m, 2H), 8.06 (d, J = 8.1 Hz, 2H), 8.18 (d, J = 8.4 Hz, 1H), 8.27–8.32 (m, 4H), 8.35–8.38 (m, 1H), 11.46 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 114.71, 118.04, 118.52, 122.39, 126.39, 126.63, 127.57, 127.75, 129.45, 132.76, 133.04, 133.11, 133.34, 133.93, 133.99, 134.70, 149.17, 154.25, 182.56 (*C*O), 185.21 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 350.0924; found, 350.0925.

4.3.15. 2-(2,5-Dimethoxyphenyl)-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (18)

Product **18** was obtained as red brown powder (yield 74%): mp 251–252 °C (EtOH) (R_f = 0.40 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3417 (NH), 1660 (C=O), 1226 (C–O). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 3.93 (s, 3H), 4.21 (s, H), 7.09 (d, J = 1.2 Hz, 2H), 7.79–7.82 (m, 2H), 8.13 (d, J = 8.1 Hz, 1H), 8.13 (s, 1H), 8.25 (d, J = 8.1 Hz, 1H), 8.29–8.36 (m, 2H), 12.37 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 56.08, 56.69, 113.13, 113.36, 116.86, 118.14, 119.98, 121.92, 124.92, 126.46, 127.54, 129.92, 132.57, 133.43, 133.70, 134.06, 134.24, 135.39, 152.20, 154.23, 155.18, 182.82 (*C*O), 184.88 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 385.1183; found, 385.1181.

4.3.16. 2-(Benzo[d][1,3]dioxol-5-yl)-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (19)

Product **19** was obtained as red brown powder (yield 81%): mp 300–301 °C (EtOH) (R_f = 0.45 at ethyl acetate/dichloromethane = 1:4). FT-IR (KBr; ν cm^{-1}) 3444 (NH), 1670 (C=O), 1257 (C–O), 1210 (C–O). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 6.11 (s, 2H), 7.00 (d, J = 7.8 Hz, 1H), 7.67 (s, 1H), 7.79–7.82 (m, 2H), 8.13 (d, J = 8.1 Hz, 1H), 8.24 (d, J = 7.8 Hz, 1H), 8.25 (d, J = 8.1 Hz, 1H), 8.29–8.36 (m, 2H), 11.18 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 101.93, 107.37, 108.96, 117.86, 121.83, 122.05, 122.78, 125.35, 126.52, 127.62, 128.40, 133.27, 133.43, 133.76, 134.08, 134.44, 148.71, 149.62, 150.56, 156.55, 182.66 (*C*O), 185.27 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 369.0867; found, 369.0887.

4.3.17. 2,2-Dimethyl-2,3-dihydro-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (20)

1,2-Diaminoanthraquinone (1.19 g, 5 mmol) was dissolved in dry acetone (100 mL) and concd sulfuric acid (0.1 mL) was added dropwise. The reaction mixture was stirred for 48 h at room temperature. The mixture was purified by column chromatography using potassium carbonate and crystallization from methanol to yield the pure compound as a purple solid in 31% yield (R_f = 0.5 at ethyl acetate/dichloromethane = 1:4). Mp 235–237 °C (MeOH) (lit: 234–237 °C).[19] FT-IR (KBr; ν cm^{-1}) 3419 (NH), 3239 (NH),1639 (CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 1.48 (s, 6H), 6.26 (d, J = 7.8 Hz, 1H), 7.37 (d, J = 7.8 Hz, 1H), 7.73–7.76 (m, 2H), 8.05 (1H, s), 8.08–8.12 (m, 2H), 8.79 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 30.18, 81.70, 104.02, 108.04, 120.99, 123.54, 126.32, 127.07, 133.41, 133.54, 134.79, 135.46, 143.05, 148.12, 179.89 (*C*O), 182.47 (*C*O). HR-MS (ESI-TOF) m/z

calcd, $[M+H]^+$ 279.1128; found, 279.1133. EI-MS m/z 278 (M^+, 8.6), 263 (100).

4.3.18. 2-Methyl-2-phenyl-2,3-dihydro-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (21)

Acetophenone (0.5 mL, 6 mmol) was added dropwise to a solution of 1,2-diaminoanthraquinone (1.19 g, 5 mmol) and concd sulfuric acid (0.1 mL) in DMF (30 mL). The reaction mixture was stirred for 72 h at room temperature. The mixture was treated with crushed ice and the resulting precipitate was collected by filtration, washed with diethyl ether and purified by crystallization from EtOH to afford desired compound as a black solid in 28% yield (R_f = 0.8 at ethyl acetate/dichloromethane = 1:4). Mp 368–371 °C (EtOH). FT-IR (KBr; ν cm^{-1}) 3348 (NH), 1671 (CO). ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 1.22 (s, 3H), 7.56–7.62 (m, 3H), 7.90–7.94 (m, 2H), 8.08 (d, J = 8.1 Hz, 1H), 8.22 (d, J = 8.1 Hz, 1H), 8.18–8.22 (m, 2H), 8.40–8.42 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 28.79, 83.56, 103.62, 109.74, 119.13, 121.35, 124.03, 126.20, 1267.76, 128.32, 128.77, 131.31, 132.99, 134.30, 134.45, 143.05, 157.25, 182.60 (*C*O), 182.89 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 341.1284; found, 341.1033.

4.3.19. 2-Mercapto-1(3)*H*-anthra[1,2-*d*]imidazole-6,11-dione (22)

1,2-Diaminoanthraquinone (1.19 g, 5 mmol) was dissolved in DMF (30 mL) and carbon disulfide (0.4 g, 5 mm) and triethylamine (3 mL) was added dropwise. The reaction mixture was continued under refluxing condition for 10 h. The reaction mixture was cooled to room temperature and treated with crushed ice and the resulting precipitate was collected by filtration, washed with diethyl ether and purified by crystallization from EtOH to afford desired compound as a brown solid in 80% yield (R_f = 0.8 at ethyl acetate/dichloromethane = 1:4). Mp 407–409 °C (EtOH). FT-IR (KBr; ν cm^{-1}) 3221 (NH), 3192 (NH),1665 (CO). ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 7.54 (d, J = 8.1 Hz, 1H), 8.02 (d, J = 8.1 Hz, 1H), 7.91–7.94 (m, 2H), 8.18–8.22 (m, 2H), 12.73 (s, 1H), 13.29 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 113.89, 115.27, 122.41, 126.26, 126.76, 126.88, 130.95, 132.89, 133.06, 134.25, 134.47, 138.19, 172.89, 181.79 (*C*O), 182.46 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 281.0379; found, 281.0389.

4.3.20. Anthra[2,1-*c*][1,2,5]thiadiazole-6,11-dione (23)

1,2-Diaminoanthraquinone (1.19 g, 5 mmol) was dissolved in dry THF (30 mL) and thionyl chloride (0.15 g, 20 mm) and triethylamine (3 mL) was added dropwise. The reaction mixture was stirred for 1 h at room temperature. The mixture was treated with crushed ice and the resulting precipitate was collected by filtration, washed with diethyl ether and purified by crystallization from EtOH to afford desired compound as a yellow solid in 74% yield (R_f = 0.8 at ethyl acetate/dichloromethane = 1:4). Mp 227–228 °C (EtOH). FT-IR (KBr; ν cm^{-1}) 1671 (CO). ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.84 (dd, J = 12.15, 6.9 Hz, 1H), 7.85 (dd, J = 13.2, 7.5 Hz, 1H), 8.33 (dd, J = 22.5, 7.2 Hz, 1H), 8.33 (dd, J = 22.5, 7.2 Hz, 1H), 8.41 (d, J = 9.3 Hz, 1H), 8.56 (d, J = 9.3 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 125.07, 126.35, 126.99, 127.34, 127.61, 132.08, 133.47, 134.15, 134.75, 135.16, 150.93, 157.99, 181.97 (*C*O), 183.31 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 267.0223; found, 267.0226. EI-MS m/z 266 (M^+, 100), 238 (64), 210 (57).

4.3.21. 2,3-Dimethylnaphtho[2,3-*f*]quinoxaline-7,12-dione (24)

Methyl vinyl ketone (0.36 g, 5 mmol) was added dropwise to a solution of 1,2-diaminoanthraquinone (1.19 g, 5 mmol) and concd sulfuric acid (0.1 mL) in DMF (30 mL). The reaction mixture was stirred for 72 h at room temperature. The mixture was treated with crushed ice and the resulting precipitate was collected by filtration, washed with diethyl ether and purified by crystallization from EtOH to afford desired compound as a black solid in 25% yield (R_f = 0.6 at ethyl acetate/dichloromethane = 1:4). Mp >400 °C (EtOH). FT-IR (KBr; ν cm^{-1}) 1671 (CO). ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 2.72 (s, 3H), 2.88 (s, 3H), 7.91–7.94 (m, 2H), 8.07 (d, J = 8.4 Hz, 1H), 8.16 (d, J = 8.4 Hz, 1H), 8.19–8.21 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 14.91, 30.74, 120.19, 125.46, 126.21, 126.26, 127.16, 128.18, 128.87, 133.01, 133.10, 134.19, 134.27, 134.42, 158.87, 162.28, 182.49 (*C*O), 183.37 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 289.0988; found, 289.0970.

4.3.22. Naphtho[2,3-*f*]quinoxaline-7,12-dione (25)

Glyoxal (40%, 0.8 g, 5 mmol) in EtOH (50 mL) was added dropwise to a solution of 1,2-diaminoanthraquinone (1.19 g, 5 mmol) in DMF (30 mL). The reaction mixture was refluxed for 16 h until water was removed. The mixture was treated with crushed ice and the resulting precipitate was collected by filtration, washed with diethyl ether and purified by from EtOH and dichloromethane to afford desired compound as a black solid in 23% yield (R_f = 0.45 at ethyl acetate/dichloromethane = 1:4). Mp 270–272 °C (EtOH). FT-IR (KBr; ν cm^{-1}) 3413, 3365, 1626 (CO). ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.82–7.87 (m, 2H), 8.29–8.36 (m, 2H), 8.48 (d, J = 8.7 Hz, 1H), 8.72 (d, J = 8.7 Hz, 1H), 8.99 (d, J = 1.5 Hz, 1H), 9.25 (d, J = 1.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 126.72, 127.05, 127.46, 130.05, 131.98, 133.77, 134.76, 135.18, 135.88, 135.93, 136.03, 145.42, 146.40, 147.77, 183.21 (*C*O), 183.61 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 261.0659; found, 261.0663. EI-MS m/z 260 (M^+, 100), 238 (73), 150 (54).

4.3.23. Naphtho[2,3-*f*]quinoxaline-2,3,7,12(1*H*,4*H*)-tetraone (26)

Oxalic acid (0.46 g, 5 mmol) was added dropwise to a solution of 1,2-diaminoanthraquinone (1.19 g, 5 mmol) and concd sulfuric acid (0.1 mL) in DMF (30 mL). The reaction mixture was refluxed for 16 h. The mixture was treated with crushed ice and the resulting precipitate was collected by filtration, washed with diethyl ether and purified by crystallization from EtOH to afford desired compound as a black solid in 30% yield (R_f = 0.25 at ethyl acetate/dichloromethane = 1:4). Mp dec. 370 °C (EtOH). FT-IR (KBr; ν cm^{-1}) 1710 (CO), 1671 (CONH). ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 7.71 (d, J = 8.0 Hz, 1H), 7.93–7.98 (m, 2H), 8.04 (d, J = 8.0 Hz, 1H), 8.17–8.24 (m, 2H), 8.99 (d, J = 1.5 Hz, 1H), 9.25 (d, J = 1.5 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 118.08, 120.52, 122.87, 126.26, 126.34, 126.78, 127.71, 128.17, 129.58, 134.48, 134.55, 135.07, 154.64 (NH*C*O), 154.73 (NH*C*O), 180.08 (*C*O), 181.07 (*C*O). HR-MS (ESI-TOF) m/z calcd, $[M+H]^+$ 293.0557; found, 293.0568. EI-MS m/z 292 (M^+), 248 (100).

4.4. Cell culture and assessment of hTERT

Non-small lung cancer cells H1299[20] 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)[21] 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 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.

4.5. 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.[22,23] 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.

4.6. Telomere repeat amplification protocol (TRAP) assays

Telomerase activity was detected by a modified version of the TRAP protocol.[24,25] 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.

4.7. SEAP assay

Secreted alkaline 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.[25,26]

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