NSC746364, NSC746365, and NSC746366: the spectra of cytotoxicity and molecular correlates of response to telomerase activity

Hsu-Shan Huang[a], Kuo-Feng Huang[c], Chia-Chung Lee[a], Chun-Liang Chen[a], Cho-Lu Li[a] and Jing-Jer Lin[b]

NSC746364, NSC746365, and NSC746366 are structurally novel 2,7-diamidoanthraquinone derivatives compared with other clinically used anticancer agents and have exhibited a unique multilog differential pattern of activity in our earlier studies. To systematically evaluate their potential anticancer activity, three selected compounds were tested for their cytotoxicity *in vitro* against 60 human cancer lines in the National Cancer Institute's anticancer drug screen as well as for dose response curves and telomerase activity. Cell growth was analyzed by the MTT assay, with differences between dose–response curves analyzed nonparametrically. Telomerase activity was detected by a modified version of the PCR-based assay and telomere repeat amplification protocol assay. To elucidate the structure–activity relationships and in-vitro anticancer activity, we correlated their activity profile [GI_{50}, total growth inhibition (TGI), and LC_{50}] in the screening system and also their effects on telomerase activity, human telomerase reverse transcriptase expression, cell proliferations, and cytotoxicity. As a result we found that NSC746364, NSC746365, and NSC746366 have potent activity with 50% net growth inhibition conferred by 0.23–16.0 μmol/l (2.08 μmol/l mean); 0.78–15.9 μmol/l (2.57 μmol/l mean); 1.38–63.1 μmol/l (3.89 μmol/l mean), respectively. Sensitive cell lines exhibit TGI and 50% lethality to NSC746364, exhibited an LC_{50} with as little as 2.82 μmol/l and TGI with as little as 0.95 μmol/l; NSC746365, exhibited an LC_{50} with as little as 3.30 μmol/l, and TGI with as little as 1.65 μmol/l; NSC746366, exhibited an LC_{50} with as little as 8.80 μmol/l; and TGI with as little as 4.06 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 unique molecular characterization, cytotoxicity, and telomerase activity profiles warrant further investigation and indicate a potential novel mechanism of anticancer action involved. *Anti-Cancer Drugs* 21:169–180 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Telomerase is the enzyme that synthesizes telomeric DNA, the terminal DNA at chromosome ends which, together with telomere-binding proteins, confers stability to chromosomes [1–3]. 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 and is therefore an attractive target for the design of anticancer agents [4]. Anthraquinone-containing extracts from different plant sources such as senna, cascara, aloe, frangula, and rhubarb have been found to have a wide variety of pharmacological activities such as anti-inflammatory, wound healing, analgesic, antipyretic, antimicrobial, and antitumor activities [5]. Binding of small molecules to quadruplexes was first studied using the classic DNA intercalator ethidium bromide and the telomere sequence of the protozoan Oxytricha [6]. Several classes of planar aromatic compounds have been shown to act as chromophore inhibitors of telomerase through stabilization of, and binding to, a folded guanine quadruplex structure [7]; anthraquinones, porphyrins, ethidium bromide derivatives, fluorenones, heteroaromatic polycyclic systems, quinolines, acridines, and telomestatins have been identified as having high affinity and selectivity for the human quadruplex telomeric sequence [8–10]. The anthracycline antibiotics (daunorubicin and doxorubicin) were introduced in clinical use more than 30 years ago for treatment of a wide variety of cancers [11]. Mitoxantrone and ametantrone are other members of the synthetic disubstituted anthraquinones developed to treat malignancies but have a narrow spectrum of antitumor activity that limits their clinical usage [12]. Therefore, many investigators consider them as highly promising lead candidates in drug design.

It was reported by Neidle and colleagues [13–17] that anthraquinones are potent human telomerase inhibitors and some members of the amidoanthraquinone class of DNA duplex intercalating agents could bind effectively

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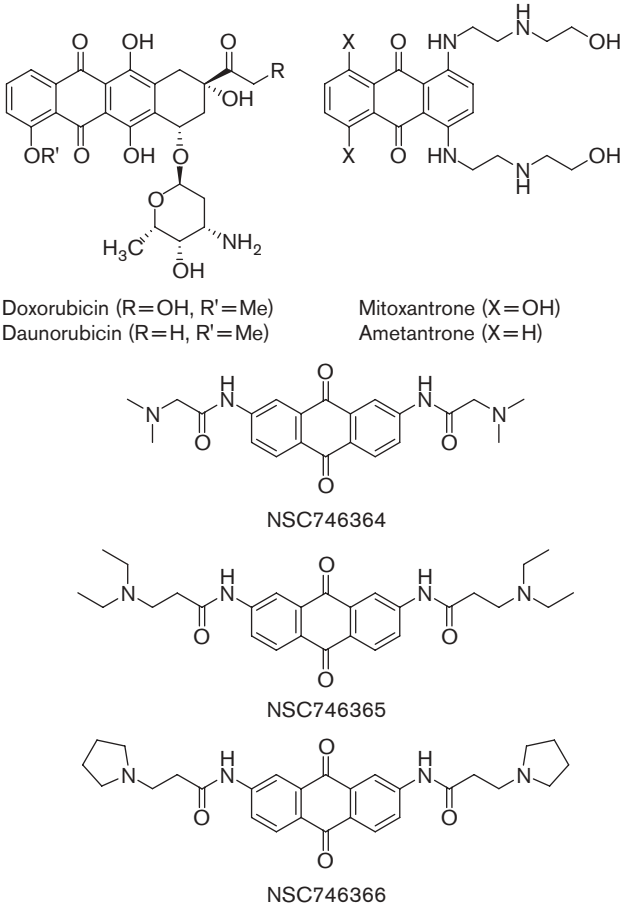
to quadruplexes and, thus, could drive the single-stranded telomere [18,19]. The DNA-binding properties of these compounds have been evaluated and it has been shown that disubstituted anthraquinones with progressively increasingly bulky substituents have a close relationship between telomerase inhibition and binding energy for the G-quadruplex structure formed by human telomeric DNA sequences [7,20]. Binding studies in solution have also shown that there is a single high-affinity binding site for a range of anthraquinone and acridine-base ligands as a stoichiometry of 1 : 1 has been determined [7,15,21]. In recent years, we have been harnessing a number of families of the telomere-targeted anthraquinone-base chromophore to generate structural novelty and diversity by biologically relevant studies [20,22–27]. It was shown that the planar anthraquinones bind onto the end of the G-quadruplex structure through n–n stacking interactions with the guanine residues and the substituents bind to the grooves of the G-quadruplex structures [13,15,20,28–30]. Among the quadruplex ligands studies, amidoanthraquinones represent one of the best small-molecules that modulate the DNA duplex versus quadruplex selectivity [13,15,31]. Thus, the anthraquinone chromophore might inhibit telomerase-dependent telomere elongation *in vitro* by stabilizing G-quadruplex structures by sequestering the DNA substrate and making it inaccessible to the telomerase [32]. In our laboratory, three lead molecules (NSC746364, NSC746365, and NSC746366) have emerged from a series of synthetic symmetrical 2,7-diamidoanthraquinone derivatives [25] which elicit potent antitumor activity against a 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, and central nervous system (CNS) cancer]. However, when the intriguing profile of tumor cell growth inhibition, cytotoxicity, and telomerase activity were initially uncovered, a molecular target became clear: these small-molecule designed telomere-targeted inhibitors caused antitumor activity and telomerase inhibition through unknown mechanisms of action. In this study, we describe the multidisciplinary evidence, approaches and experiments undertaken to elucidate structure–activity relationships (SARs) in the antitumor activity of several 2,7-disubstituted amidoanthraquinones as molecular targets. Molecules in this series displayed a highly unusual pattern of selectivity in the NCI Developmental Therapeutics Program (DTP) in-vitro screen of 60 human-derived cancer cell lines. The molecular approach, evidence of antitumor activity and telomerase activity has also been obtained, and the implications of these findings are discussed (Fig. 1).

Fig. 1



Chemical structures of bioactive chromophoric anthraquinone-base antitumor agents, NSC746364, NSC746365, and NSC746366.

Materials and methods

Chemicals

The synthesis and chemical characterization of NSC746364, NSC746365, and NSC746366 have been described previously [25,33]. Compounds such as those described here, which interact selectively with NCI DTP's in-vitro 60 cell line screen and inhibit telomerase, are potentially useful as inhibitors of the proliferation of cells that require telomerase to maintain telomere length for continued growth. As telomerase is found almost exclusively in tumor cells, compounds with the type of structure described in this study may be useful as novel antitumor agents.

The National Cancer Institute's anticancer drug screen

As a primary screening, five selected compounds were submitted to the National Cancer Institute (NCI) cell line screen for evaluation of their anticancer activity [34]. From the data analysis it follows that approximately 95% of the actives from the 60 cell line screen can be identified. The detailed methods used for the 60 cell line panel have been described elsewhere [35–41]. In brief, cellular protein levels were determined after 48 h of drug exposure by sulforhodamine B colorimetry. Through the

use of a time 0 cell control, cell growth can be determined for each cell line, thus allowing calculations of the 50% growth inhibitory concentration (GI_{50}), the total growth inhibition (TGI), and the 50% lethal concentration (LC_{50}). Comparison to plates not exposed to drug permits determination of the concentration and times of exposure conferring 50% net growth inhibition (GI_{50}), TGI, and 50% cell kill (LC_{50}). These data are then plotted as mean bar graphs and as dose–response curves. By these criteria, three compounds reported were active and passed on for evaluation in the full panel of 60 human tumor cell lines.

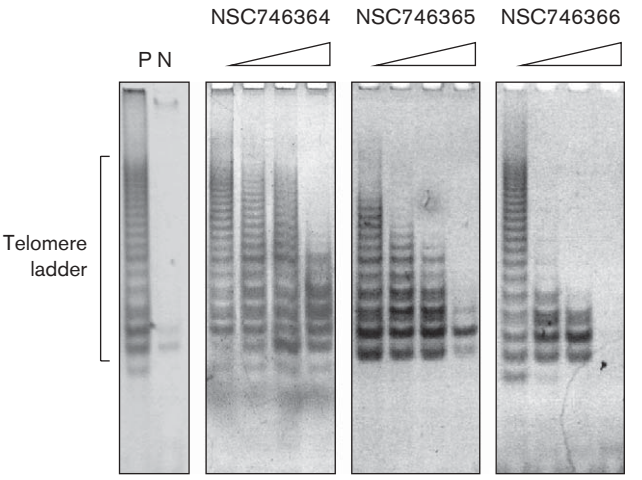
Growth inhibition

The panel is organized into nine subpanels representing diverse histologies: leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and CNS [35]. Cells were seeded into 96-well microtiter plates at a density of 5×10^3 per well and allowed 24 h to adhere before drugs were introduced. Serial drug dilutions were prepared in medium immediately before each assay. The test compounds were dissolved in dimethylsulfoxide (DMSO) and evaluated using five concentrations at 10-fold dilutions, the highest being 10^{-4} mol/l and the others being 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} mol/l. Viable cell masses at the time of drug addition (time 0), and after 72 h of drug exposure were determined by cell-mediated MTT reduction. MTT was added to each well and plates incubated at 37°C for 4 h to allow reduction of MTT by viable cell dehydrogenases to an insoluble formazan product. Well supernatants were aspirated and cellular formazan solubilized by the addition of DMSO/glycine buffer. Cell growth and drug activity were determined by measuring absorbance at 550 nm using an Anthos Labtec systems plate reader. The concentration that produced 50% growth inhibition (GI_{50}) compared with a DMSO control, TGI, or 0% growth, compared with a DMSO control, and the concentration that produced the death of 50% of the cells present at the start of the experiment LC_{50} were determined. Figures 2–4 report the results obtained with this test expressed as the $-\log$ of the molar concentration that inhibited the cell growth by 50% (pGI_{50}), or that caused total cytostasis (pTGI, TGI), or that killed half the cells (pLC_{50}) when compared with the values of untreated control cells. For the calculation of the MG_MID values, insensitive cell lines are included with the highest test concentration.

Cell culture and assessment of human telomerase reverse transcriptase

Non-small lung cancer cells H1299 [42] 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 human telomerase reverse transcriptase (hTERT) immortalized hTERT-BJ1 [43] was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin, 1 mmol/l sodium pyruvate, and 4 mmol/l 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 in which the expression of hTERT could be monitored by a reporter system, a ~3.3 kbp DNA fragment ranging from −3338 to +1 bp of the hTERT gene was subcloned upstream to a secreted alkaline phosphatase (SEAP) gene select 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.

Fig. 2

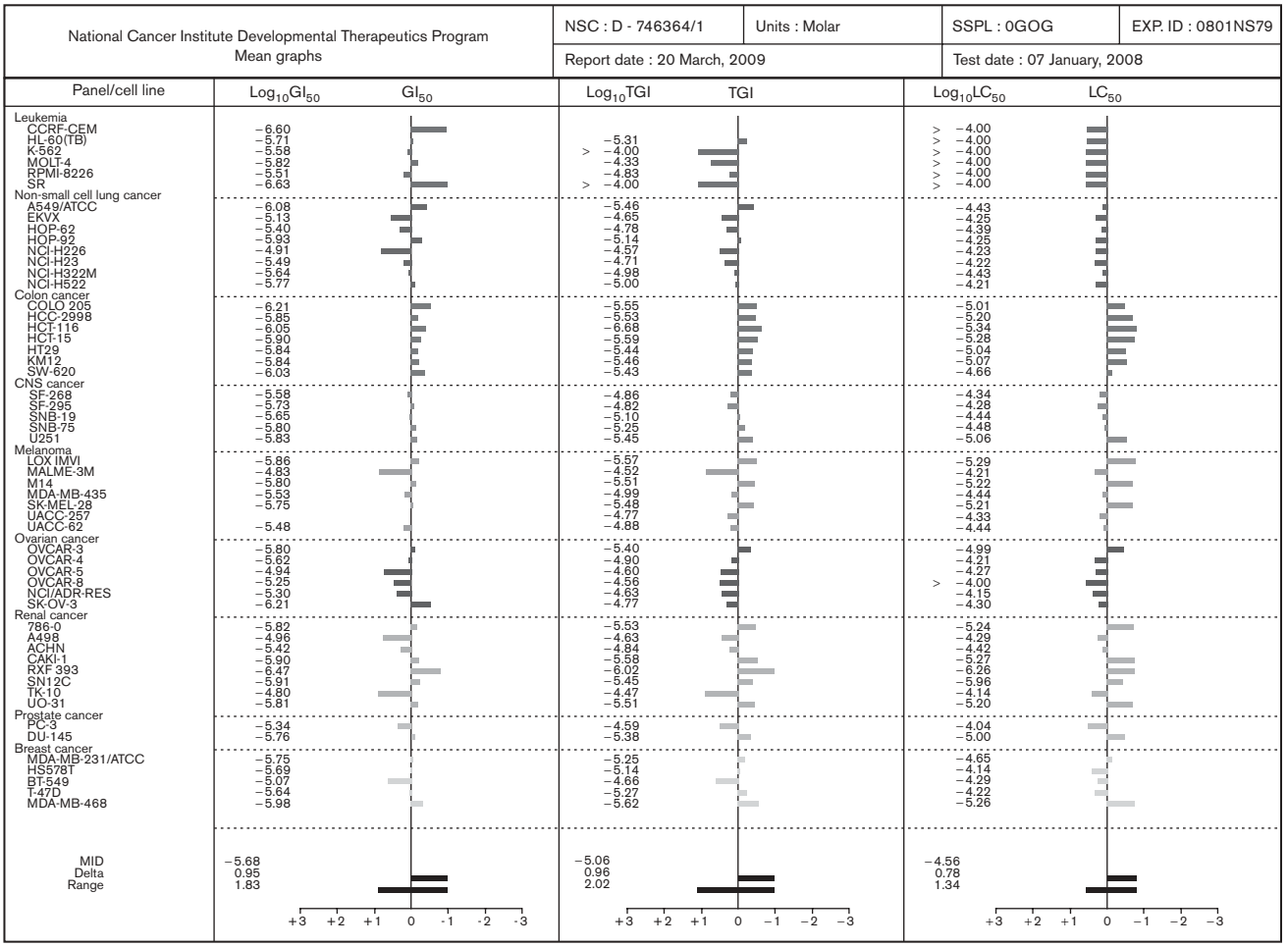


Averaged mean graphs for testing NSC746364 in the NCI Developmental Therapeutics Program's in-vitro 60 cell line screen on three separate occasions. The figure provides a graphic and tabular listing of the molar drug concentrations (log units) conferring GI_{50}, total growth inhibition (TGI), and LC_{50} for each cell line. The response of each cell line relative to the mean of all cell line response is depicted by a horizontal bar extending either to the right (more sensitive) or to the left (less sensitive) of the mean (vertical line) for each index of activity (GI_{50}, TGI, and LC_{50}). The length of each bar is proportional to the cell line sensitivity relative to the mean in log units. Mean bar graph plots permit comparisons of individual cell line responses, as well as a 'fingerprint' of all cell line responses for a particular test compound [41], growth curves for NSC746364 in the NCI-60 cell line panel. Dose–response curves were generated as described in Materials and methods. N, negative control; NCI, National Cancer Institute; P, positive control.

Cytotoxicity assay

The tetrazolium reagent MTT [3-(4,5-di-methylthiazol)-2,5-diphenyltetrazolium bromide; USB Corporation, Ohio, USA] was designed to yield a colored formazan upon metabolic reduction by viable cells [44,45]. Approximately 2×10^3 cells were plated onto each well of a 96-well plate and incubated in 5% CO_2 at 37°C for 24 h. To assess the in-vitro cytotoxicity, each compound was dissolved in DMSO immediately before the experiments and the solutions were diluted into the complete medium before addition to cell cultures. Test compounds were then added to the culture medium at the various

Fig. 3



Averaged mean graphs for the testing of NSC746365 in the NCI Developmental Therapeutics Program's in-vitro 60 cell line screen on three separate occasions. CNS, central nervous system; NCI, National Cancer Institute; TGI, total growth inhibition.

designated concentrations. After 48 h, 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 absorbence at 550 nm was measured using an enzyme-linked immunosorbent assay reader.

Telomere repeat amplification protocol assays

Telomerase activity was detected by a modified version of the telomere repeat amplification protocol (TRAP) [46–48]. Telomerase products were resolved by 10% polyacrylamide gel electrophoresis and visualized by staining with SYBER Green (Sigma-Aldrich Co., California, USA). 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 a Bio-Rad protein assay kit (Bio-Rad, USA) using bovine serum albumin standards.

SEAP assay

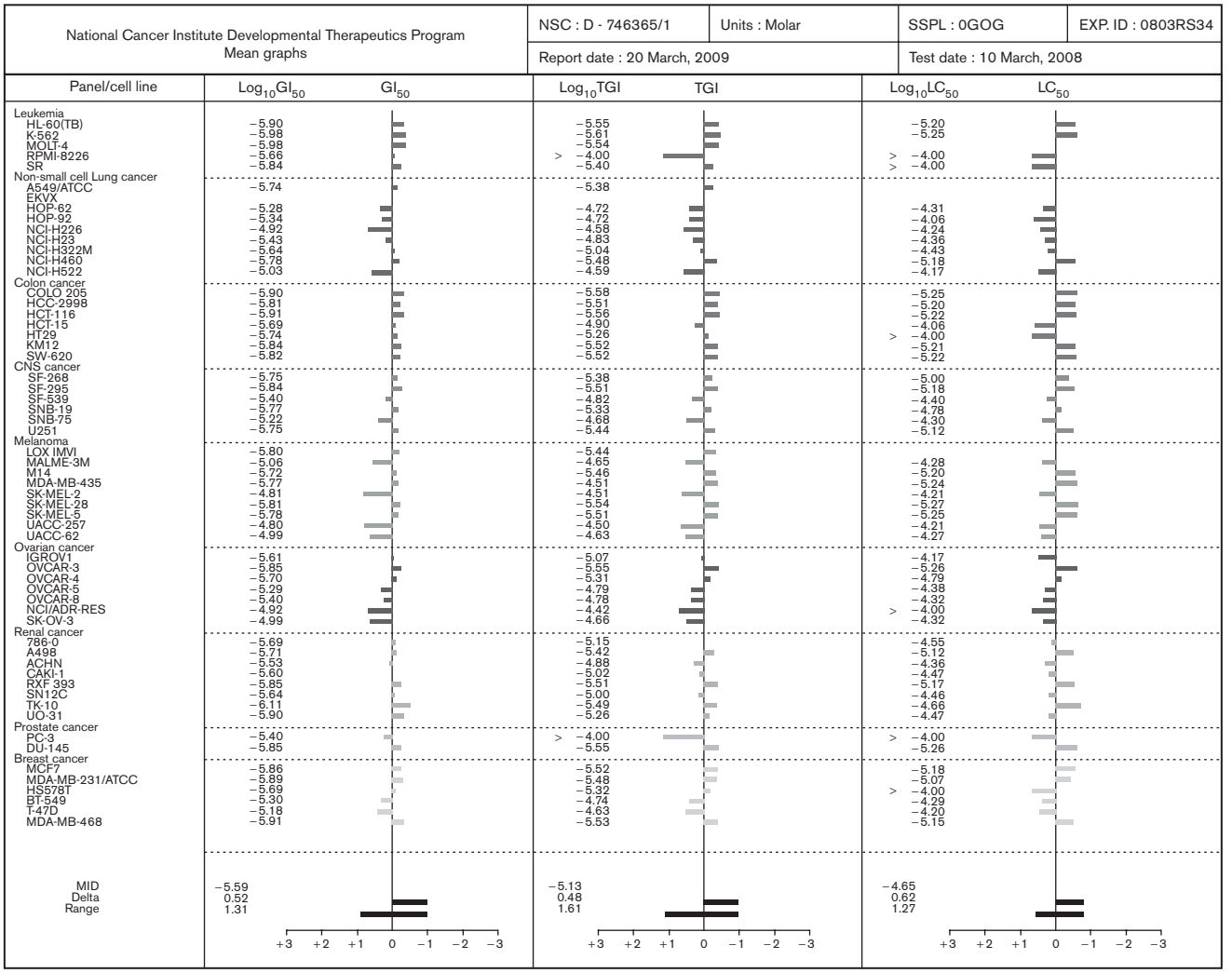
SEAP was used as the reporter system to monitor the transcriptional activity of hTERT. Here, about 10^4 cells each were grown in 96-well plates and incubated at 37°C for 24 h and changed with fresh media. Varying amounts of drugs were added and cells were incubated for another 24 h. Culture media were collected and heated at 65°C for 10 min to inactivate heat-labile phosphatases. An equal amount of SEAP buffer (2 mol/l diethanolamine, 1 mmol/l $MgCl_2$, and 20 mmol/l l-homoarginine) was added to the media and *p*-nitrophenyl phosphate was added to a final concentration of 12 mmol/l. Absorptions at 405 nm were taken, and the rate of absorption increase was determined [49].

Results

In-vitro antitumor activity

To understand the unique antiproliferative activity pattern of test compounds in the NCI-60 cell line screen,

Fig. 4



Averaged mean graphs for the testing of NSC746366 in the NCI Developmental Therapeutics Program's in-vitro 60 cell line screen on three separate occasions. CNS, central nervous system; NCI, National Cancer Institute; TGI, total growth inhibition.

we computed SARs between the three derivatives: NSC746364, NSC746365, and NSC746366. They exhibited dose-dependent inhibition of proliferation in all 60 cancer cell lines. As shown in Figs 2–4, the cytotoxicity elicited by the three analogs 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. It may be appreciated from these representations of the data that the three analogs represent the most potent derivatives from the series of 2,7-disubstituted amidoanthraquinones. As with a series of compounds, the mean of each of the endpoints across all 60 cell lines is calculated. For each cell line, the difference between the GI_{50} for that cell line and the mean GI_{50} across all cell lines is also calculated. As shown in Fig. 2, for NSC746364 the average concentration required to inhibit GI_{50} was 2.09 μmol/l with a range of 0.23 μmol/l (leukemia cancer SR) to 16.0 μmol/l (renal cancer TK-10). Figure 2 shows the dose–response curves of all the cell lines panel. The concentration at which cell growth is inhibited by 50% represents the GI_{50}.

As shown in Fig. 3, for NSC746365 the average concentration required to inhibit GI_{50} was 2.57 μmol/l with a range of 0.78 μmol/l (renal cancer TK-10) to 15.9 μmol/l (melanoma, UACC-257). As shown in Fig. 4, for NSC746366 the average concentration required to inhibit GI_{50} was 3.89 μmol/l with a range of 1.38 μmol/l (leukemia, CCRF-CEM) to 63.1 μmol/l (leukemia, SR). In addition, more than 1 μmol/l (10–6 mol/l) was required to achieve LC_{50} in the majority of human tumor cell lines; cell lines sensitive to NSC746364 exhibited an LC_{50} with

as little as 2.82 μmol/l (colon cancer, COLO 205) and TGI with as little as 0.95 μmol/l (renal cancer, RXF-393). Cell lines sensitive to NSC746365 exhibited an LC_{50} with as little as 3.30 μmol/l (non-small cell lung cancer, NCI-460) and TGI with as little as 1.65 μmol/l (non-small cell lung cancer, NCI-460). Cell lines sensitive to NSC746366 exhibited an LC_{50} with as little as 8.80 μmol/l (non-small cell lung cancer, NCI-460) and TGI with as little as 4.06 μmol/l (non-small cell lung cancer, NCI-460). The GI_{50} value is the negative $\log_{10}$ of the concentration required to inhibit the growth of that cell line by 50% (relative to untreated cells). TGI is the negative $\log_{10}$ minimum concentration that causes TGI, and LC_{50} reflects the negative $\log_{10}$ concentration needed to kill 50% of the cells [37]. Results may be unexpected, the $>10^3$ range in the GI_{50}, the $>10^3$ range in the TGI, and the $>10^2$ range in the LC_{50} drug concentrations among cell lines together, with the mean-graph pattern of cell line sensitivity [50], suggest that this test compound confers a multilog, differential effect upon cell lines rather than exerting a nonspecific cytotoxicity in which most cell lines would show very similar GI_{50}, TGI, and/or LC_{50} indices. NSC746364, NSC746365, and NSC746366 exhibited dose-dependent inhibition of proliferation in all 60 cancer cell lines. Of the three, they were the most potent compounds during all the selected evaluations, with an average GI_{50} of 2.09 μmol/l for NSC746364, 2.57 μmol/l for NSC746365, and 3.89 μmol/l for NSC746366, respectively. All these data are summarized in Table 1 and were used for further analysis.

Telomerase activity

We evaluated the effects of NSC746364, NSC746365, and NSC746366 on telomerase inhibition using a PCR-based telomerase assay, and TRAP assay. We also designed and expressed SEAP, MTT, and TRAP assays to provide a suitable target for telomerase activity assay [25]. Results are summarized in Table 2 and all three derivatives showed MTT and SEAP inhibition activity in the 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 antiproliferation capacity in tumor cells, we expected that analysis of NSC746364, NSC746365, and NSC746366 might yield further insight into this relationship.

As shown in Fig. 5, NSC746364, which contains a simple side chain of an isopropylamino-acetamido [$–CH_2NHCH(CH_3)_2$] unit appended to the substituent in the 2,7-positions, showed high selective inhibition activity towards 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 in the receptor site. The results of an SARs study indicated that the torsion angle between the central chromophore ring and the chloroacetamido substituent, and the bond length of the linker at the anthraquinone moiety, might be important for G-quadruplex-binding activity. We also evaluated the effects of NSC746364, NSC746365, and NSC746366 on telomerase activity using a PCR-based telomerase assay and TRAP assay. Comparative in-vitro telomerase inhibitory screening of NSC746364, NSC746365, and NSC746366 provided some intriguing observations of inhibitory profiles. It is interesting to note that most of these compounds with side chains affected telomerase inhibitory activity. Thus, in addition to indicating an anticancer function, our findings also 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 [25]. As shown in Fig. 1, NSC746364 with side chains of $–NHCO\text{-}CH_2NHCH(CH_3)_2$, NSC746365 with side chains of $–NHCO\text{-}(CH_2)_2N(C_2H_5)2$, and NSC746366 with side chains of $–NHCO\text{-}(CH_2)_2NC_4H_8$ show inhibition of G-quadruplex-induced telomerase at IC_{50} levels of 15.5, 14.2, and 6.7 μmol/l, respectively. NSC746366 with a side chain pyrrole ring (IC_{50} 6.7 μmol/l) is the most potent telomerase inhibitor in this series of compounds. It is surprising that NSC746365, with $–NHCO\text{-}(CH_2)_2N(C_2H_5)_2$ side chains, has similar differences in cytotoxicity which might have a significant effect on its interaction with DNA. It is especially notable that NSC746366 had both cytotoxicity and telomerase activity whereas for cytocidal effects this difference was significant. However, it had a significant influence on the spectrum of antiproliferative activity.

Discussion and conclusion

Herein we described the in-vitro anticancer profile and telomerase activity of NSC746364, NSC746365, and NSC746366. Determining the mechanism of action or identifying the molecular target of a chemical with anticancer activity is highly desirable. Various test methods are currently available for determining the mechanisms of action of chemicals, such as methods based on (1) NCI-60 cell lines screen assay, (2) dose–response curves model, (3) in-vitro testing results model, and (4) mean graphs model and telomerase activity assay for expressed SEAP, MTT, and TRAP assays. Nevertheless, determination of the mechanisms of action of pharmacologically active chemicals is still a difficult and challenging task. This telomere-targeted inhibitor with a broad-spectrum antitumor activity approach enables mechanism-oriented evaluation of anticancer drugs. For example, we can evaluate cell toxicity *in vitro* by determining the GI_{50}, TGI, and LC_{50} across a panel of

Table 1 In-vitro anticancer activity of NSC746364, NSC746365, and NSC746366 in the NCI's 60 human cancer cell lines

	NSC746364 (μmol/l)			NSC746365 (μmol/l)			NSC746366 (μmol/l)		
Panel/cell line	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Leukemia									
CCRF-CEM	0.25	–	>100	–	–	–	1.38	4.14	>100
HL-60(TB)	1.95	4.94	>100	1.25	2.79	6.26	8.40	>100	>100
K-562	2.61	>100	>100	1.06	2.44	5.63	–	–	–
MOLT-4	1.51	46.4	>100	1.06	2.89	–	8.77	>100	>100
RPMI-8226	3.12	14.8	>100	2.18	>100	>100	2.14	6.03	>100
SR	0.23	>100	>100	1.43	4.00	>100	63.1	>100	>100
Non-small cell lung cancer									
A549/ATCC	0.82	3.49	36.9	1.44	1.83	4.14	3.77	40.6	>100
EKVX	7.37	22.4	56.4	3.05	–	–	8.80	77.5	>100
HOP-62	4.00	16.7	40.9	1.73	5.21	19.0	3.94	19.5	>100
HOP-92	1.16	7.17	56.0	1.47	4.57	19.1	2.38	34.8	>100
NCI-H226	12.4	27.2	59.3	6.56	11.9	26.2	7.67	28.9	98.6
NCI-H23	3.21	19.4	60.7	1.53	3.73	14.8	4.38	23.8	>100
NCI-H322M	2.29	10.5	37.5	2.85	2.29	9.09	2.34	10.1	>100
NCI-460	–	–	–	1.90	1.65	3.30	1.88	4.06	8.80
NCI-H522	1.71	10.1	61.2	1.40	9.34	25.7	3.21	34.9	>100
Colon cancer									
COLO 205	0.61	2.82	2.82	1.26	2.65	5.57	1.63	6.29	>100
HCC-2998	1.43	2.98	6.25	1.55	3.12	6.31	2.83	12.6	39.7
HCT-116	0.88	2.10	4.58	1.24	2.74	6.04	1.93	6.86	>100
HCT-15	1.25	2.56	5.27	2.05	12.6	88.0	5.15	22.0	67.2
HT29	1.46	3.63	9.03	1.84	5.55	>100	2.98	>100	>100
KM12	1.43	3.50	8.56	1.44	3.00	6.22	2.13	7.14	>100
SW-620	0.93	3.74	21.7	1.51	3.01	5.97	1.87	>100	>100
CNS cancer									
SF-268	2.62	13.9	45.7	1.79	4.21	9.92	3.27	>100	>100
SF-295	1.87	15.3	52.4	1.44	3.10	6.66	2.52	12.3	>100
SF-539	–	–	–	3.96	15.1	40.0	3.42	12.8	44.9
SNB-19	2.25	7.85	36.6	1.68	4.69	16.7	2.46	16.7	>100
SNB-75	1.60	5.59	33.3	5.99	21.0	49.7	6.03	22.4	66.1
U251	1.48	3.58	8.67	1.76	3.66	7.61	2.73	11.3	48.1
Melanoma									
LOX IMV1	1.39	2.68	5.18	1.57	3.60	–	1.67	–	>100
MALME-3M	14.8	30.1	61.0	8.77	22.5	52.7	4.24	21.8	>100
M14	1.59	3.10	6.05	1.91	3.45	6.24	6.14	28.3	>100
MDA-MB-435	2.96	10.2	36.5	1.68	3.09	5.69	3.50	18.0	>100
SK-MEL-2	–	–	–	15.4	30.6	61.1	19.4	45.4	>100
SK-MEL-28	1.80	3.34	6.22	1.53	2.86	5.35	3.94	19.3	82.4
SK-MEL-5	–	–	–	1.68	3.06	5.59	3.48	16.6	76.2
UACC-257	–	17.0	46.3	15.9	31.3	61.5	8.28	25.7	73.9
UACC-62	3.30	13.1	36.2	10.2	23.3	53.2	5.91	82.2	>100
Ovarian cancer									
IGROV1	–	–	–	2.43	8.54	67.6	3.77	22.9	>100
OVCAR-3	1.59	4.00	10.2	1.42	2.81	5.56	3.29	14.3	>100
OVCAR-4	2.39	12.6	61.6	2.00	4.90	16.3	3.73	16.6	64.7
OVCAR-5	11.4	24.9	54.3	5.08	16.1	41.6	4.97	44.0	>100
OVCAR-8	5.61	27.3	>100	3.97	16.6	48.3	3.79	>100	>100
NCI/ADR-RES	5.06	23.6	70.2	12.1	37.8	>100	62.7	>100	>100
SK-OV-3	0.61	17.0	50.4	10.2	22.1	47.9	2.98	33.5	>100
Renal cancer									
786-0	1.52	2.96	5.77	2.04	7.15	28.5	3.88	20.7	>100
A498	10.9	23.6	51.1	1.94	3.83	7.56	2.47	9.13	53.0
ACHN	3.81	14.3	37.8	2.96	13.2	43.4	3.53	18.1	>100
CAKI-1	1.27	2.62	5.42	2.50	9.54	34.2	4.02	29.9	>100
RXF 393	0.34	0.95	5.51	1.41	3.08	6.77	4.90	34.0	>100
SN12C	1.22	3.58	11.0	2.28	10.1	34.5	3.12	12.9	35.9
TK-10	16.0	34.0	72.5	0.78	3.20	13.8	2.58	8.63	93.2
UO-31	1.54	3.12	6.33	1.27	5.43	33.7	4.15	54.7	>100
Prostate cancer									
PC-3	4.54	25.7	90.5	3.98	>100	>100	2.62	11.1	>100
DU-145	1.74	4.16	9.90	1.41	2.79	5.53	3.53	14.6	51.4
Breast cancer									
MCF7	–	–	–	1.37	3.02	6.63	1.42	4.35	>100
MDA-MB-231/ATCC	1.79	5.66	22.3	1.30	3.33	8.55	2.49	6.65	31.2
HS 578T	2.04	7.17	72.5	2.05	4.84	>100	3.76	>100	>100
BT-549	8.50	22.0	51.0	5.00	18.2	51.7	4.05	33.1	>100
T-47D	2.28	5.35	59.9	6.62	23.3	63.2	3.38	18.7	>100
MDA-MB-468	1.04	2.38	5.47	1.24	2.98	7.14	2.52	40.4	>100

CNS, central nervous system; NCI, National Cancer Institute; TGI, total growth inhibition.

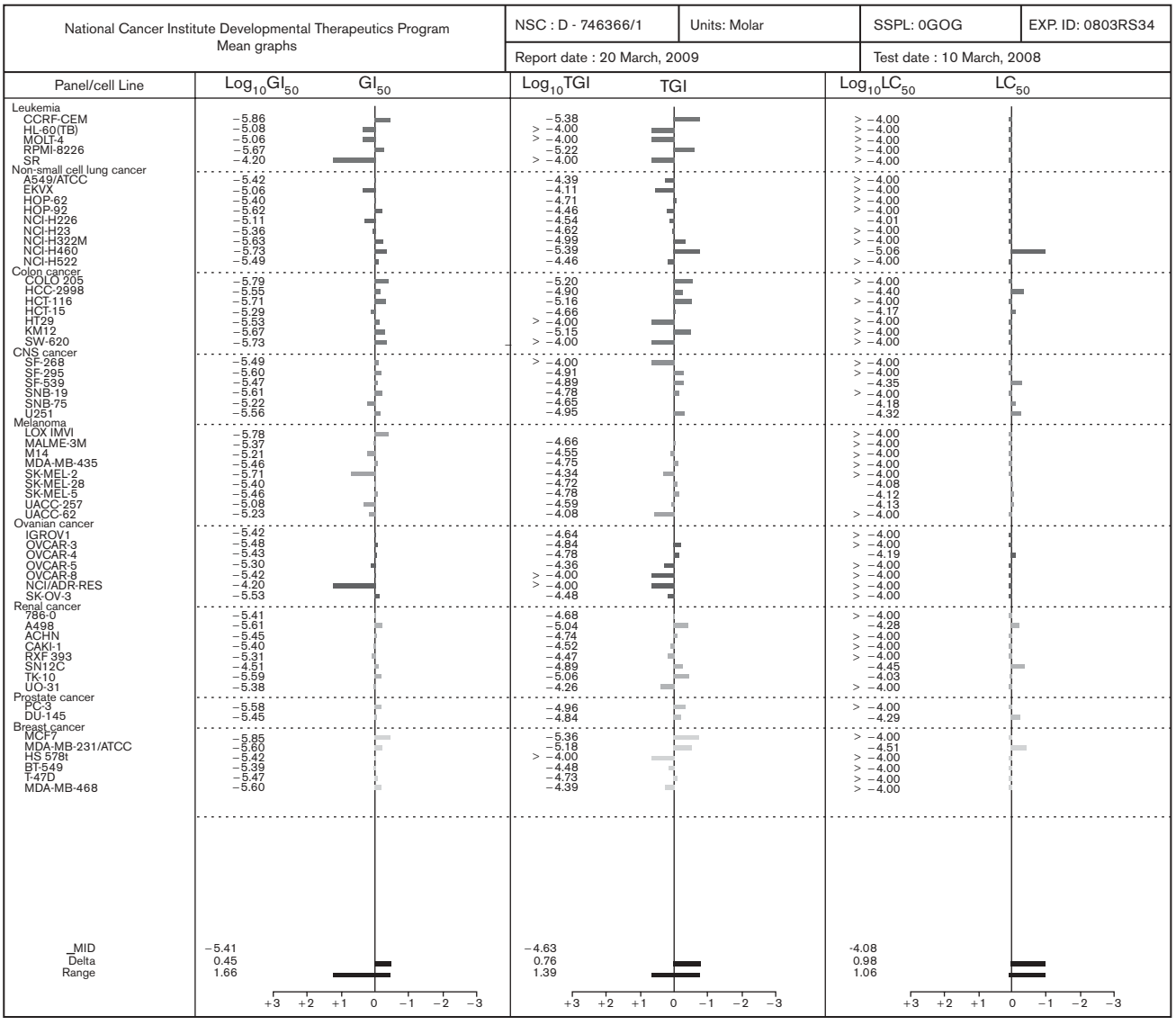
Table 2 Effects of NSC746364, NSC746365, and NSC746366 on hTERT expression and telomerase activity

Cell type (inhibition concentration, IC_{50}, μmol/l)				
	H1299		BJ1	
Compounds	MTT	SEAP	MTT	SEAP
NSC746364	6	6	8	55
NSC746365	9	7	34	10
NSC746366	19	6	66	26

hTERT, human telomerase reverse transcriptase; SEAP, secreted alkaline phosphatase.

NCI-60 cell lines. Thus, in this study, we have examined the potential of NSC746364, NSC746365, and NSC746366, and our data show that the molecular targets or evaluate the action mechanisms of the test compounds by comparing cell growth inhibition profiles across the panel of these compounds. However, we found that doxorubicin and mitoxantrone did not belong to the same mechanisms, which consisted of telomere-targeting anticancer drugs. At present, we do not understand the reason; however, there is a possibility that NSC746364, NSC746365, and NSC746366 have other action mechanisms,

Fig. 5



Inhibition of telomerase activity in which the telomere repeat amplification protocol 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 concentrations of test compounds were 1, 5, 10, 20 μmol/l, respectively. As the internal control (IC) shares one oligonucleotide with the reaction, the IC products became apparent when telomerase activities were inhibited. TGI, total growth inhibition.

which may have made the fingerprint of doxorubicin and mitoxantrone different from those of other telomere-targeting drugs.

Telomerase is a reverse transcriptase that prevents the mortality checkpoints evoked by programmed telomere shortening during each round of cell division [51]. The hTERT is highly expressed (approximately 90%) in stem cells, germ cell lines, and most human tumors, and its inhibition and G-quadruplex stabilizers represent both strategies for the development of selective anticancer drugs [46,52–55]. Collectively, the telomerase inhibitors illustrate how repositioning of existing drugs could complement *de novo* drug development. A current trend in chemical modifications has appended the examples that have already been tested. Efforts were directed toward extending the approach to the synthesis of other biologically interesting telomerase inhibitors. Could telomerase inhibitors be repositioned as anticancer agents? Our results in the NCI-60 cell line panel show that some selective amidoanthraquinones have a wide spectrum of activity, inhibiting the proliferation of 60 cancer cell lines derived from nine different tumor types. This is consistent with earlier reports showing that telomerase inhibitors are effective in other model systems [23,25–27,56]. Of the five 2,7-disubstituted amidoanthraquinones evaluated [25], only three were effective, suggesting that the chemical requirements for telomerase inhibition and anticancer activity are not identical. NSC746364, NSC746365, and NSC746366 were rationally designed to stabilize the G-quadruplex structure of telomeres and their interaction with G-quadruplex DNA. Earlier studies confirmed that G-quadruplexes as targets for drug design, and G-quadruplex structures of DNA represent a potentially useful target for anticancer drugs [22,57,58]. Molecules used in the present assay and their numbering are given in Fig. 1. At present, among different strategies actively pursued to inhibit telomerase activity in cancer cells, the development of G-quadruplex stabilizers has emerged as a highly promising approach [8,9,55]. As telomerase reverse transcriptase activity depends on the 3′ single-stranded telomeric DNA end acting as a primer, small-molecule structures that bind and stabilize the folded quadruplex form of the primer can inhibit enzyme action [22]. As noted in these companion compounds, NSC746364, NSC746365, and NSC746366 are chemically novel and biologically unique in their mechanism of action and in-vitro pattern of activity. In this study, we present evidence that NSC746364, NSC746365, and NSC746366 possess a number of pharmacologically desirable properties. First, NSC746364, NSC746365, and NSC746366 have a multilog differential pattern of activity in the NCI-60 cell line screen. These results suggest that they may possess biologically unique mechanism(s) of action. Of particular note, there are significant differences in the mean graph pattern of activity for NSC746364, NSC746365, and NSC746366, compared with that of mitoxantrone and doxorubicin, and from which the 2,7-disubstituted amidoanthraquinone structures were derived. Interestingly, they all contain a unique amine with one or two carbon space linker moieties that could provide a structural basis for the differences in potency between these and other telomerase inhibitors. Our data clearly extend the biological mechanisms used by mitoxantrone and doxorubicin.

In addition, NSC746364, NSC746365, and NSC746366 exhibit a broad spectrum of in-vitro antitumor activity and were highly efficacious in each of the nine tumor xenograft models studied. From a general structure–activity standpoint, these tricyclic planar anthraquinone pharmacophores demonstrate that structural variations can result in significant changes in specificity and potency with regard to anticancer activity. The amidinium moiety is known to contribute to the stabilization of DNA recognition elements through electrostatic and hydrogen bonding interactions [59]. Therefore, hydrogen bonds are frequently used as recognition elements due to their directionality and are also an attractive approach to the biological activity [60]. Therefore, many classes of drugs have substantial curvature, high DNA affinity, binding to the minor-groove or major-groove, and interference with DNA-associated enzymes (e.g. telomerase). The binding affinities and specificities observed suggest that the incorporation of a variety of moieties will lead to substances that interact with DNA targets. This approach greatly expands the utility of the substituents and related chromophore for the construction of drugs in general. Therefore, it is desirable to design telomerase inhibitors by means of various anticancer parameters.

The anthraquinone moiety was introduced into the central position of the modified DNA by connection with the non-nucleic backbone instead of the corresponding nucleoside [61]. Small molecules (e.g. anthraquinone, etc.) that stabilize the possible G-quadruplex structure of telomeres [29,62] might act by either inhibiting telomerase activity by locking the single-stranded telomere substrate into a quadruplex structure or triggering telomere uncapping by generating an abnormal telomere structure [63–65]. Although many drug discovery programs screen for novel small-molecule telomerase inhibitors, no appropriate candidate has been found to be a sufficiently potent and specific inhibitor, even though many chemical compounds have been screened. There have been many recent significant developments in the telomere/telomerase fields of research, but there are still many unclear in our understanding. Telomerase-targeted therapies are a promising and novel approach to cancer therapeutics that could lead to effective interventions for the treatment of cancer with minimal side effects [55]. Among the NCI and telomerase activity studied, cellular effects produced by several small-molecule compounds have now been studied in detail, and indicate that classic

telomerase inhibition does occur, with subsequent telomere length attrition. Carefully evaluated cytotoxicity and telomerase inhibition of amidoanthraquinones as telomerase inhibitors might afford a unique opportunity to probe its roles in NCI-60 cell line dose–response and telomerase activity. On the basis of the accumulated in-vitro biological data, NSC746364, NSC746365, and NSC746366 are currently being developed by the US NCI DTP for screening evaluation. The three were chosen because they have been reported to be more biologically active than the others after one-dose assay and found to be very potent. They also exhibit a multilog differential pattern of activity in which some cell types were markedly more sensitive over several logs of drug concentration.

There is additional evidence that NSC746364, NSC746365, and NSC746366 were symmetrical substituents based on the anthraquinone chromophore in which the linker consists of two amido subunits separated by a planar tricyclic ring system. Appropriately substituted amidoanthraquinones are effective G-quadruplex stabilizers, but there is not sufficient information at present on the possible modification of G-quadruplex recognition and telomerase inhibition produced by the position of the amide bond [22]. Furthermore, anthraquinones, porphyrins, ethidium bromide derivatives, fluorenones, heteroaromatic polycyclic systems, quinolines, acridines, and telomestatins have been identified with high affinity and selectivity for the human quadruplex telomeric sequence [8–10]. It was first reported by Neidle *et al.* [13–17] that anthraquinone analogs are potent human telomerase inhibitors. Previous work by their laboratory demonstrated that disubstituted anthraquinones with progressively increasingly bulky substituents have a close relationship between telomerase inhibition and binding energy for the G-quadruplex structure form by human telomeric DNA sequences [7]. To date, a number of families of compounds have been developed and their telomerase activity and cytotoxicity have been studied extensively [20,22–25]. In this investigation, we continue to focus our attention on the role of NSC746364, NSC746365, and NSC746366 and to understand the basis of pharmacophore selectivity. The optimal number of atoms between the amido nitrogen atom and terminal side chain nitrogen is two, which corresponds to ~ 4 Å distance [25]. An SAR study was also conducted on a number of amidoanthraquinone derivatives [18,23,24,26,27,66–70]. The chemical and biological activities of anthraquinones are greatly affected by its various substituents of the planar tricyclic ring system, which considered as aglycon analogs of anthracycline antibiotics. As 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.

In conclusion, the rationally designed 2,7-disubstituted amidoanthraquinones, NSC746364, NSC746365, and NSC746366, are the lead clinical candidates in a novel class of compounds that produce unique telomerase activity and cytotoxicity. In the course of these experiments, the data in this study indicate that the compounds exhibit potent and differential in-vitro activity against cancer cells compared with normal hematopoietic cells. On the basis of these pharmacologically desirable properties and the broad-spectrum in-vitro index of activity (GI_{50}, TGI, and LC_{50}) reported, NSC746364, NSC746365, and NSC746366 are currently undergoing more detailed preclinical pharmacology and toxicology studies through our laboratory. Additional evidence that the dose-dependent inhibition of proliferation in all 60 cancer cell lines and cytotoxicity were derived from experiments showing that with their GI_{50}, TGI, and LC_{50}. Collectively, these studies show that NSC746364, NSC746365, and NSC746366 can induce pharmacological and biochemical features of cell proliferation and viability in the NCI-60 cell line panel. Our data clearly extend the biological evidence used by the three selective compounds. Although 2,7-substituted amidoanthraquinones have previously been reported to cause antiproliferation and cytotoxicity [25], our studies here show that nonapoptotic death were also responsible for cytotoxicity in multiple cell lines. As a whole, these data support the conclusion that all the tested molecules (NSC746364, NSC746365, and NSC746366), share a common target in telomerase and cytotoxicity that may be a novel and less toxic modality of cancer therapy. These results confirm that the amide bond direction in amidoanthraquinones would favor G-quadruplex recognition, DNA-interactive and targeting telomerase in preventive and/or curative therapy. Thus, this approach using the NCI-60 cell lines panel is useful not only in predicting the action mechanisms of novel chemicals but also in evaluating their anticancer activity.

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