Novel Piperazinediones as Antitumor Agents

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Abstract. *Chemical modification of dipeptide mimetic azatyrosine led to a series of piperazinediones. Thirteen piperazinediones were synthesized and tested for their anticancer activity. This series of piperazinedione compounds exhibited potent anticancer activities against human disease-oriented cancer cell lines in NCI60 cancer screening (National Cancer Institute, USA). Among them, four leads (compound **10**, **18**, **21**, and **22**) exhibited in vitro tumor growth suppression, reducing tumor cell growth to 45.7%- 56.3%, and exhibited broad-spectrum activities. Compound **18**, with 50% cancer cell growth inhibition (GI_{50}) <10 nM in 45 cell lines from the NCI, was selected as the lead for further mechanism of action studies. The mechanism of action was predicted by the COMPARE algorithm and confirmed by experiments as inhibition of tubulin polymerization which inhibits the formation of microtubules.*

The need for cancer chemotherapy continues to grow, with a 13% annual growth rate. Among such therapies, small molecule-based ones continue to represent the backbone of anticancer treatment, with a share of $50 billion USD (65%) in 2008, expected to reach $90 billion USD (55%) in 2013 (1). Most importantly, new chemotherapy agents with selective toxicity are still the unmet medical need for cancer treatment. A series of azatyrosinamides synthesized in professor Hui-po Wang's laboratory in Taipei Medical University (Taipei, Taiwan) demonstrated selective cytotoxicity against Rat sarcoma (*ras*)-transformed NIH3T3 cells and ras mutation-associated human cancer cell lines (2, 3). Structural modification of these compounds by transporter approach for the purpose of improving their pharmacokinetic profiles led to the production of dipeptide mimetic azatyrosine analogs (4, 5). Further modification of the dipeptide mimetic azatyrosinamides led to a series of piperazinediones as cyclized azatyrosinamides (6, 7). This report describes the design and synthesis of piperazinedione analogs, the *in vitro* anticancer activities and the mechanism of action (MOA) prediction from Developmental Therapeutics Program (DTP), National Cancer Institute (NCI)/National Institute of Health (NIH) database to wet-lab confirmation.

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Key Words: Piperazinediones, NCI-60 anticancer screening, *in vivo* antitumor activity, COMPARE algorithm, azatyrosinamide, tubulin polymerization inhibitor, mechanism of action prediction, DTP, NCI/NIH.

Materials and Methods

Chemistry. Piperazinedione compounds were synthesized based on literature methods (8-10) and were patented (US 6635649 B2, US 20120232088 A1). All compounds were confirmed by ^{1}H-nuclear magnetic resonance and high-resolution mass spectroscopy.

Pharmacology. In vitro cytotoxicity: In house and by Developmental Therapeutic Program (DTP), National Cancer Institute (NCI)/National Institute of Health (NIH): Compounds were tested for cytotoxicity against AGS (ATCC: CRL-1739; Bioresource Collection and Research Center, Hsinchu City, Taiwan, ROC) with 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) assay (11). Cancer cells were treated with 1 μM of test compounds. Dimethyl sulfoxide (DMSO) was used as a vehicle control and six wells were prepared for each compound. After treatment, the supernatant was carefully aspirated and 150 μl of DMSO was added to each well. The absorbance was measured at 590 nm.

Compounds with better anticancer activities were submitted to DTP, NCI/NIH for panel *in vitro* screening (12, 13), following standard procedures (http://dtp.nci.nih.gov/docs/misc/common_files/submit_ compounds.html). The DTP screening system consists of approximately 60 cell lines of major human tumors, and the tumor growth inhibition activity of test compounds were monitored by the sulforhodamine B (SRB) assay (12). Each test compound was evaluated at five 10-fold dilutions (10^{-4}M to 10^{-8}M), producing a

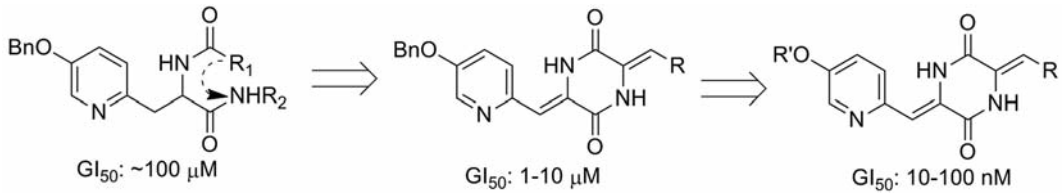


Figure 1. *Design of piperazinediones for optimization of anticancer activity of azatyrosinamides. GI_{50}: 50% cancer cell growth inhibition.*

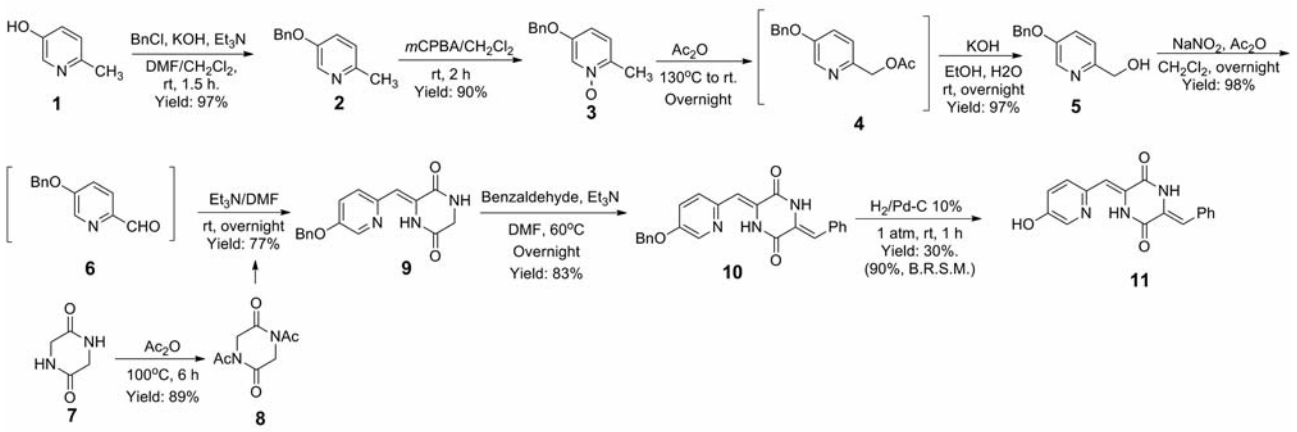


Figure 2. *Synthesis of piperazinedione compounds to intermediate compound 11. BnCl: Benzyl chloride; KOH: potassium hydroxide; ET_3N: triethylamine; DMF: N,N-dimethylformamide; CH_2Cl_2: dichloromethane; rt: room temperature; mCPBA: meta-chloroperoxybenzoic acid; Ac_2O: acetic anhydride; EtOH: ethanol; Pd-C 10%: palladium on charcoal (10%); B.R.S.M: based-on recovered starting material.*

dose–response curve, and the 50% growth inhibition concentration (GI_{50}) was determined. After study completion, crude data were obtained as a text file and a mean graph (14) plotted by DTP which represented the *in vivo* potency. The tumor-suppressive activity was denoted as pGI_{50} in which $pGI_{50}=-\log GI_{50}$.

COMPARE algorithm. After we submitted the compounds to DTP, a cancer chemotherapy National Service Center (NSC) number was generated after approval by the DTP. When the study was completed, we submitted the NSC number to the COMPARE website (http://dtp.nci.nih.gov/compare-web-public_compare/login.do), and found the reference compound with most similar GI_{50} profile across the NCI60 cell lines, defined as the highest Pearson product-moment correlation coefficient (Pearson's r). The core assumption is that when two compounds share a similar NCI60 profile, they may share similar a MOA (13,14).

Tubulin polymerization inhibition. Inhibition of tubulin polymerization was measured using the tubulin polymerization assay kit (Cytoskeleton Inc. Denver, CO, USA) according to the manufacturer's directions. Briefly, 300 μg of purified bovine brain tubulin were incubated with tubulin assembly buffer (80 mM Na-Pipes, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM GTP and 10% glycerol) and individual drug in a final volume of 100 μl. The polymerization of purified tubulin into microtubules was determined by monitoring the absorbance at 340 nM at 37˚C in a Varioskan™ Flash Multimode Reader (Thermo Electron Corp., Finland).

Results

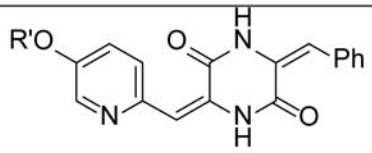
Chemistry. Literature procedures were followed with modification for the preparation of picolinaldehyde intermediate **6** (8-10). Key intermediate compound **9** was synthesized by aldol-condensation reaction of intermediate **6** with **8**. Compound **10**, the di-substituted piperazinedione was prepared by condensation of compound **9** with benzaldehyde (9). Hydrogenolysis of intermediate **10** provided compound **11** (Figure 2). This compound served as an intermediate for the preparation of a series of piperazinedione analogs for activity studies.

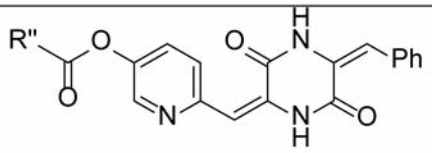
We designed and synthesized different piperazinedione analogs to optimize anticancer activity. We tried methylene bridging (compounds **12-20**) and ester linkage (compounds **21-24**). Other substituents were designed based on electron density change of the terminal BnO group (Figure 3).

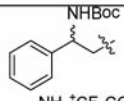
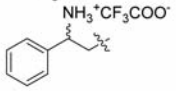
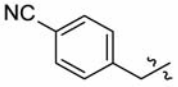
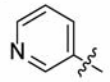
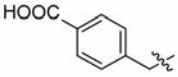
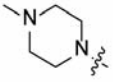
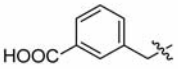
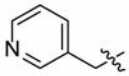
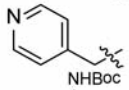
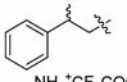
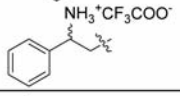
Pharmacology. Compounds **10-24** were subjected to *in vitro* anticancer screening on an AGS stomach cancer cell line by the MTT assay (11) (Table I).

Compounds with highest tumor cell growth suppression (**10, 18, 21, 22**) were submitted to DTP, NCI/NIH for *in vitro* NCI60 panel screening (Figure 4) (Table II) (12, 13).

Table I. *Tumor cell growth suppression of AGS human stomach cancer cell line by piperazinedione analogs.*





Compound no.	R′	Growth percentage (at 1 μM)	Compound no.	R′′	Growth percentage (at 1 μM)
10	Ph	45.7%	**21**		54.9%
11	H	57.7%	**22**		56.3%
12		57.8%	**23**		67.1%
14		73.2%	**24**		75.6%
16		67.2%			
17		51.8%			
18		51.0%			
19		77.7%			
20		63.2%			

We further analyzed the NCI60 inhibition profile using the COMPARE algorithm provided by DTP, NCI/NIH to search for reference compounds from NCI public synthetic compound database. The COMPARE service allows compounds with most similar NCI60 profiles to be found (13, 14), which means they have similar MOA to our compounds. The reference compounds identified as having high similarity (Pearson's r>0.6) were input into SciFinder® (provided by American Chemical Society, Columbus, Ohio, USA) and referring literature sought. We identified possible MOA of our piperazinedione compound **10** (Table III) from the referring literature. The same approach was used to identify the possible MOA of three other compounds (Table IV).

Table II. *NCI60 growth inhibition constant of compounds **10**, **18**, **21**, and **22**.*

	Compound No.			
	10	**18**	**21**	**22**
Mean GI_{50} (nM)	363.08	52.48	398.11	364.74
Median GI_{50} (nM)	169.82	<10	162.18	117.49

The possible MOAs of the four piperazinedione compounds were related to inhibition of tubulin polymerization. The most potent, compound **18**, was

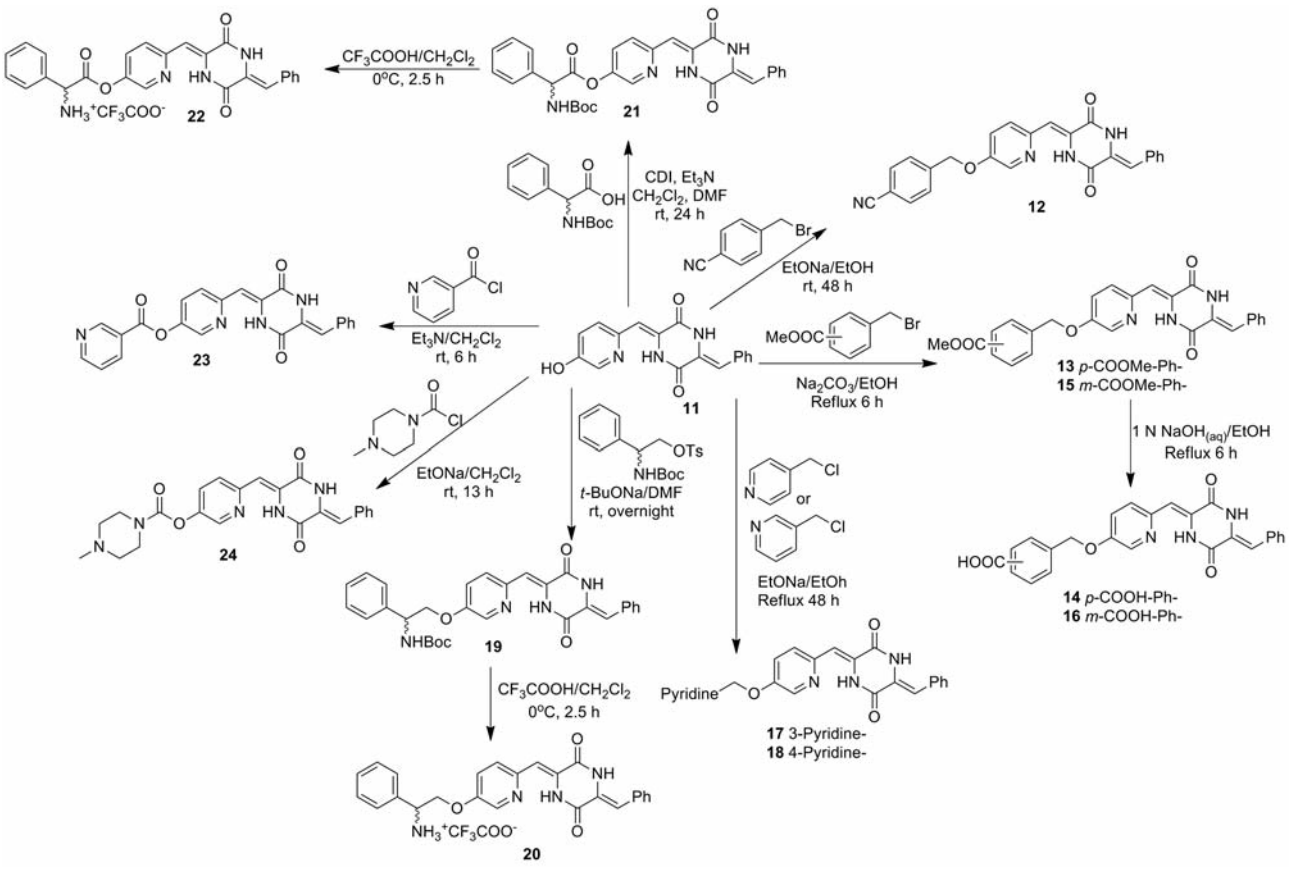


Figure 3. *Synthesis of piperazinedione analogs from intermediate compound **11**. EtONa: Sodium ethoxide; rt: room temperature; Boc: tert-butyloxycarbonyl; t-BuONa: sodium tert-butoxide; DMF: N,N-dimethylformamide; CDI: carbonyldiimidazole; Et_3N: triethylamine.*

Table III. *COMPARE result and possible mechanism of action of compound **10**.*

Compound	Cytotoxicity in NCI60 panel	COMPARE result					Possible MOA
		COMPARE ranking	NSC number	Pearson's r	MOA from literature	Reference	
10	Mean pGI_{50}: 6.44	2	679,019	0.797	Tubulin polymerization inhibitor	(15-16)	Tubulin polymerization inhibitor
	Median pGI_{50}: 6.77	3	750,945	0.790	N/A*	N/A*	
	Range: 4.00-7.40	4	711,616	0.787	Tubulin binder	(17)	
	Most susceptive:	5	344,270	0.749	Binding to tubulin colchicine binding site	(18)	
	Melanoma, MDA-N	6	673,622	0.743	Tubulin polymerization inhibitor	(19)	
		7	667,466	0.731	Tubulin polymerization inhibitor	(20)	
		8	751,382	0.729	Tubulin binding	(21)	
		9	708,781	0.727	Similar to temozolomide	(22)	
		10	736,994	0.714	Binding to tubulin colchicine binding site	(23)	

*No literature available on SciFinder®.

selected for tubulin polymerization inhibition study to confirm this finding (Figure 5). Compound **18** exhibited significant tubulin polymerization inhibition, similar to standard tubulin polymerization inhibitors such as colchicine, not to tubulin de-polymerization inhibitors such as taxol. The predicted possible MOAs from COMPARE, DTP, NCI/NIH were confirmed by *in vitro* studies.

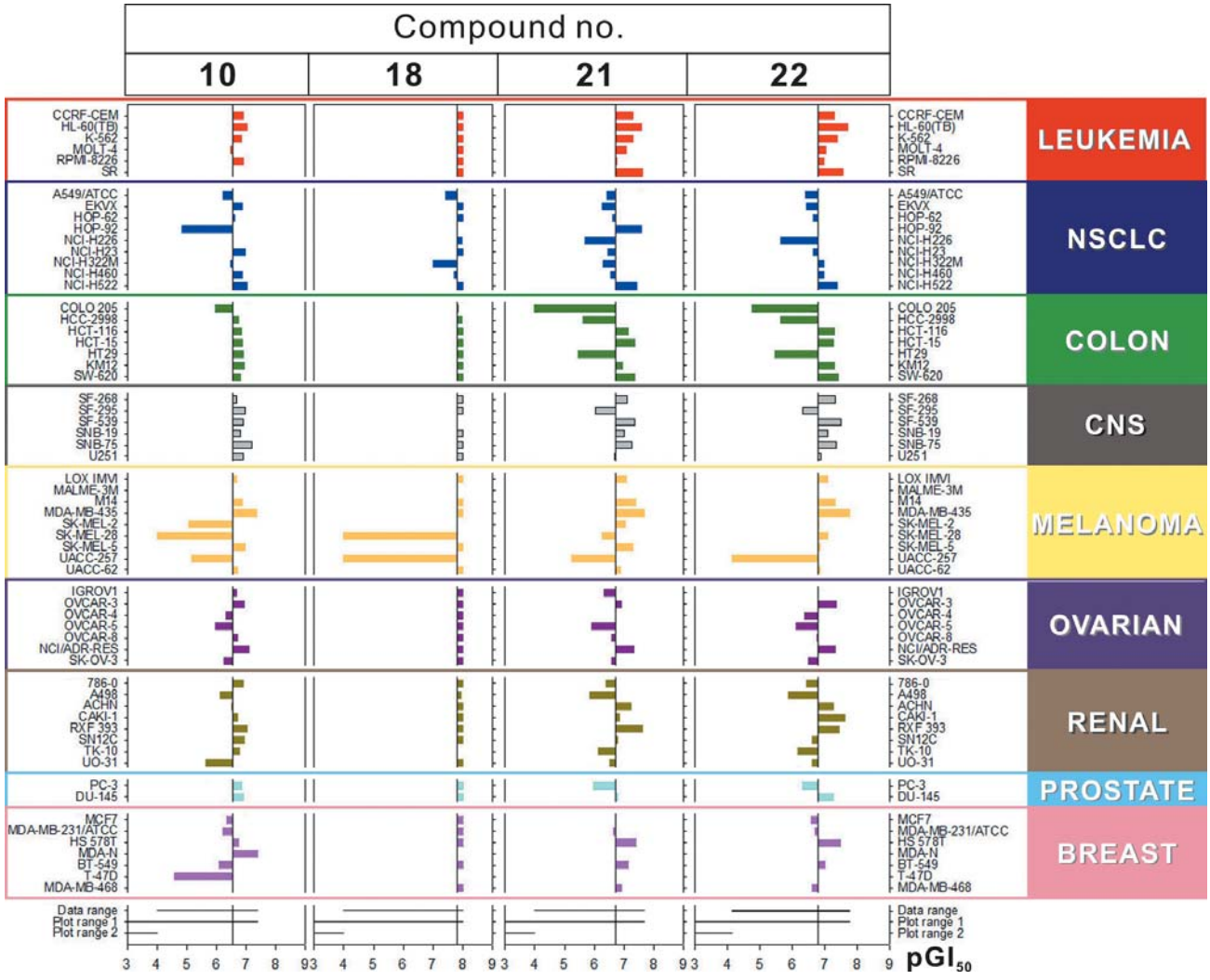


Figure 4. *The in vitro anticancer activity of compounds **10**, **18**, **21** and **22**. Data are from Developmental Therapeutic Program, National Cancer Institute.*

Discussion

Piperazinedione compounds were designed as cyclized dipeptide mimetics of azatyrosinamide. These compounds are suggested to be tubulin polymerization inhibitors which showed good *in vitro* tumor cell suppression. In synthesizing compound **6** (Figure 2), we used $NaNO_2/Ac_2O$ oxidation instead of MnO_2 or chromate-containing oxidation agents to yield compound **6**. This process can reduce the use of toxic chemicals, and overcome common problems in the oxidation reaction of the benzylic position (10).

Comparing the *in vitro* tumor growth inhibition (Table II), an aromatic ring in the terminal R' or R'' group may be essential for activity (compound **10, 12, 17, 18, 23**), since the sp3 heterocyclic ring may reduce the activity (compound **24**), as may a hydrophilic group (compound **14, 16**). The four selected piperazinedione compounds exhibited better activity against leukemia, CNS cancer, but not melanoma (Figure 4). This may explain why tubulin- targeting agents were not used in the management of melanoma (24).

We also compared the *in vitro* activity with approved oncological drugs (Figure 3; data from DTP, NCI/NIH http://dtp.nci.nih.gov/branches/dscb/oncology_drugset_explanation.html). The cytotoxicity profile of most oncological drugs lies between pGI_{50} 6-8 (1 μM $<GI_{50}<$10 nM) except for taxol and doxetaxel; but compound **18** had a pGI_{50} of 8 in 45 out of 60 cell lines. Compared to traditional cytotoxic agents such as DNA cross-linkers, alkylating agents, anti-metabolites and DNA chelators, compound **18** showed better potency in suppressing tumor growth. However, compared to kinase inhibitors and hormone therapy, pGI_{50} of 4-6 (100 μM $<GI_{50}<$1 μM), this suggested that

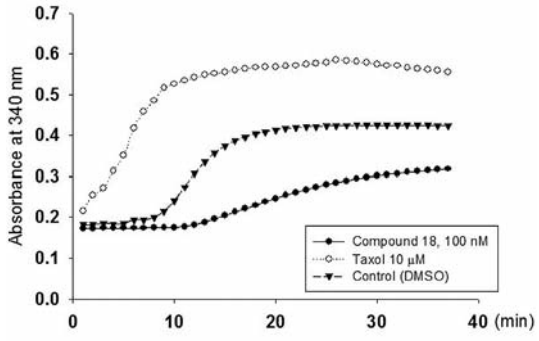


Figure 5. *Inhibition of tubulin polymerization (proportional to absorbance) by compound* ***18***.

Table IV. *Possible mechanism of actions of selected piperazinedione compounds.*

Compound (NSC no.)	Cytotoxicity profile in NCI60 panel	COMPARE result		Possible MOA
		NSC no.	r value	
10	Mean pGI_{50}: 6.44	679,019	0.797	Tubulin
(711,720)	Median pGI_{50}: 6.77	750,945	0.790	polymerization
	Range: 4.00-7.40	711,616	0.787	inhibitor
	Most susceptive:	344,270	0.749	
	Melanoma, MDA-N	673,622	0.743	
		667,466	0.731	
		751,382	0.729	
		708,781	0.727	
		736,994	0.714	
18	Mean pGI_{50}: 7.75	711,616	0.867	Tubulin
(750,945)	Median pGI_{50}: 8	706,462	0.810	polymerization
	Range: 4.00-8.00	164,850	0.798	inhibitor
	Most susceptive:	292,222	0.791	
	45 cell line	711,720	0.790	
	GI_{50} <10 nM	713,210	0.760	
		663,321	0.758	
		667,446	0.731	
		750,212	0.727	
21	Mean pGI_{50}: 6.72	750,949	0.822	Tubulin
(750,948)	Median pGI_{50}: 6.83	675,003	0.801	polymerization
	Range: 4.00-7.67	748,553	0.772	inhibitor
	Most susceptive:	659,853	0.761	
	Melanoma,	748,541	0.746	
	MDA-MB-435	106,969	0.721	
		681,683	0.720	
		641,150	0.719	
		653,008	0.717	
22	Mean pGI_{50}: 6.80	750,948	0.822	Tubulin
(750,949)	Median pGI_{50}: 6.97	106,969	0.767	polymerization
	Range: 4.15-7.77	680,185	0.759	inhibitor
	Most susceptive:	675,003	0.753	
	Melanoma,	698,666	0.746	
	MDA-MB-435	165,897	0.718	
		676,190	0.697	
		676,187	0.697	
		341,931	0.696	

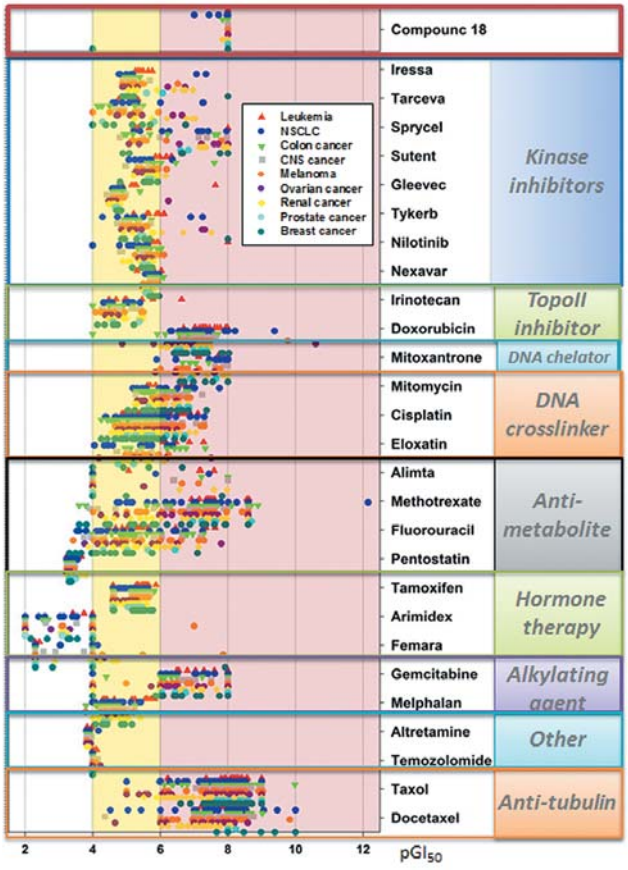


Figure 6. *Comparison of cytotoxicity of compound* ***18*** *to commonly used anticancer drugs. Each data point represented the* pGI_{50} *(*pGI_{50}*=–log*GI_{50}*;* GI_{50}*: 50% cancer cell growth inhibition) of a cell line, and the color of the data point represents the cell type. NSCLC: non-small cell lung cancer; CNS: central nerve system.*

compound **18** may have cytotoxicity-related adverse effects, such as neutropenia, which remain to be investigated.

Following the report by Shoemaker (13), we used the COMPARE service to discover reference compounds with similar NCI60 profiles, defined as Pearson's r>0.6. In our findings (Tables III-IV), tubulin binding was suggested as our MOA. This was confirmed by an *in vitro* tubulin polymerization inhibition study, in which compound **18** led to significant tubulin polymerization inhibition, similar to other tubulin-targeting agents such as colchicine. This serves as a good example of using a large amount of data to predict the MOA of a compound, a method which can be used for many other compounds with unknown MOA, such as natural products.

Conclusion

Compound **18** showed promising *in vitro* tumor suppression, superior to many traditional chemotherapeutic agents. The MOA of compound **18** was predicted from the database

provided by DTP, NCI/NIH and then proven by wet-lab experiment to be inhibition of tubulin polymerization. Further formulation design for pharmacokinetic/pharmacodynamic optimization, pharmacokinetic and toxicity evaluation (ADMET) will be conducted.

Acknowledgements

This study was funded by grant 102-EC-17-A-20-S1-196 from the Ministry of Economic Affairs of Taiwan. The Authors declare that there is no conflict of interests regarding the publication of this article. All authors are/were working in school (Taipei Medical University) or government funded research institute (Industrial Technology Research Institute). The patent owners (patent assignee) are Taipei Medical University and National Taiwan University. This is a follow-up study of patent US6635649 B2 and 20120232088 A1. Study results in this article have not been disclosed in these two patents.

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Received March 19, 2014
Revised June 8, 2014
Accepted June 10, 2014