Anticancer Activity of 5-Benzylidene-2-Phenylimino-1, 3-Thiazolidin-4-one (BPT) Analogs

S. Wu[†], W. Guo[†], F. Teraishi[†], J. Pang[‡], K. Kaluarachchi[¥], L. Zhang[†], J. Davis[†], F. Dong[†], B. Yan[†,§] and B. Fang[†,*]

[†]*Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA;* [‡]*Pharmaceutical Development Center, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA;* [¥]*Experimental Therapeutics Department, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA;* [§]*School of Pharmaceutical Sciences and Institute of Environmental Sciences, Shandong University, Jinan, Shandong, China*

Abstract: We recently identified two compounds of 5-benzylidene-2-phenylimino-1,3-thiazolidin-4-one (BPT) analog, 5-(4-methylbenzylidene)-2-phenylamino-1,3-thiazolidin-4-one (MMPT) and 5-(2,4-dihydroxybenzylidene)-2-phenylimino-1,3-thiazolidin-4-one (DBPT), that can effectively induce apoptosis in cancer cells but not in normal cells, independently of P-glycoprotein status. To further investigate the antitumor activity of BPT analogs, we obtained 18 commercially available analogs of BPT and synthesized 7 analogs in our lab, and analyzed their antitumor activity in various cancer cells, including paclitaxel- and vinorelbine-sensitive and -resistant human lung cancer cells. Two of the compounds were more potent than MMPT or DBPT in induction of apoptosis in certain cancer cell lines and remained tumor selective. Seven compounds did not induce any cytotoxic effects in any of the cell lines tested at the highest concentration tested (31 μM). The other compounds induced cytotoxic effects in some cancer cells but not in others or were less potent than MMPT and DBPT. Cell uptake studies showed that analogs that effectively induced cell killing in paclitaxel- and vinorelbine-resistant cells could be taken up easily by those cells despite their high levels of P-glycoprotein expression. These data further demonstrate that thiazolidinone analogs are not P-glycoprotein substrates and could be useful for treatment of P-glycoprotein overexpressing refractory cancers.

Key Words: 5-Benzylidene-2-Phenylimino-4-Thiazolidin-4-one (BPT) analog, Apoptosis, Cytotoxic effects, Chemical core structure, LC-MS, P-glycoprotein, Multidrug-resistance, Antitumor activity.

INTRODUCTION

Multidrug resistance (MDR), a phenotype of cross-resistance to multiple drugs with diverse chemical structures, is one of the major reasons for failure of anticancer chemotherapy and can be caused by a variety of mechanisms [1-2]. The most well-documented mechanism is overexpression of the MDR-1 gene which encodes a 170-kD P-glycoprotein, a member of the ATP-binding cassette transporter family [1-3]. P-glycoprotein is a transmembrane ATP-dependent drug efflux pump that is expressed in a wide variety of tumors and in some normal tissues with barrier function, including epithelia of the liver, kidney, and intestine and capillary endothelial cells in the brain, ovary, and testis [2-4]. It binds to many hydrophobic drugs, including taxanes, vinca alkaloids, epipodophylotoxins, anthracyclines, and actinomycin-D, and prevents their intracellular accumulation by increasing their efflux out of cells, leading to multidrug resistance [1-2].

Efforts have been made to overcome MDR by inactivating P-glycoprotein activity through P-glycoprotein inhibitors (such as verapamil, cyclosporin A, tamoxifen, and LY-335 979) [5-7], antisense oligos [8], ribozyme [9-10], or small interfering RNA [11]. Although a growing body of evidence shows that the MDR phenotype can be reversed in cultured cells by these approaches, clinical trials that examined the ability of P-glycoprotein inhibitors to sensitize MDR-positive tumors to agents such as vinblastine revealed either no appreciable activity [7] or no definitive conclusion [2,6] Moreover, inhibition of P-glycoprotein may lead to myelosuppression and neurological toxicity [2,6], presumably because P-glycoprotein plays an important role in the excretion of cytoxines, which prevents toxic effects to normal tissues. Therefore, the discovery of new compounds that are not substrates of P-glycoprotein and are effective against drug-resistant cells but spare normal human cells is an important step for cancer therapy.

By screening a chemical library from Chembridge Corporation, we identified two novel synthetic agents, 5-(4-methylbenzylidene)-2-phenylamino-1,3-thiazolidin-4-one (MMPT) and 5-(2,4-dihydroxybenzylidene)-2-phenylimino-1,3-thiazolidi-4-one (DBPT), which induce cytotoxic effects in cancer cells but not in normal human fibroblasts or human bone marrow mesenchymal stem cells [12-13]. We have shown that MMPT and DBPT have potent antitumor effects on non-small cell lung cancer cells or colon cancer cells that overexpress G-glycoprotein and are resistant to paclitaxel and vinorelbine. MMPT and DBPT have similar chemical

*Address correspondence to this author at the Department of Thoracic and Cardiovascular Surgery, Unit 445, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA; Tel: (713) 563-9147; Fax: (713) 794-4669; E-mail: bfang@mdanderson.org

1573-4064/06 $50.00+.00

structures. *In vivo* studies showed that MMPT suppressed tumor growth without noticeable toxicity. Interestingly, one compound of BTP analogs induced no cytotoxicity in either cancer cells or normal cells. Nevertheless, it remains unclear whether other analogs of BPT except MMPT and DBPT may induce cytotoxicity in cancer cells and whether MMPT or DBPT mediated cytotoxic effects in paclitaxel-resistant cells correlate with their intracellular accumulation. To determine the structure-activity relationship of BPT analogs, we evaluated antitumor activity of 27 compounds, which included MMPT and DBPT, in various cancer cells. We also assessed intracellular accumulation of some of these compounds in paclitaxel- or vinorelbine-resistant cancer cells. Our results showed that the analogs can be taken up effectively by MDR1-overexpressing cells.

RESULTS AND DISCUSSION

Chemical Structure of BPT Analogs

To test whether BPT analogs can also induce tumor-selective cytotoxic effects, we obtained 25 compounds that have chemical structures similar to those of MMPT or DBPT (ChemBridge Corporation). Seven of them (BPT-12, BPT-15, PBT-23 to BPT-27) were synthesized in our laboratory. The intermediates and the final products were separated and purified by silica gel column chromatography based on polarity of the compounds. The purity and molecular identity were determined by high-performance liquid chromatography-mass spectrometry (LC-MS).The compounds have purity of ≥95%, and their molecular weights matched the predicted mass. For some BPT analogs, we verified their chemical structure by nuclear magnetic resonance (NMR) analysis. Fig. (**1A**) shows the core chemical structure of BPT and the synthetic route to BPT analogs. For convenience, we refer to the two aromatic cyclic-ring structures, where modification occurs, as ring A and ring B (Fig. **1A**). The modifications at different positions of these two rings that characterize the analogs are summarized in Table **1**. They are divided into two groups based on the position of substituents in ring A and ring B. Group I contains 15 analogs that have substituents in the ring A only. Group II contains 12 analogs that have substituents in ring B or both rings A and B.

Cytotoxic Effects of BPT Analogs

The cytotoxic effects of 27 thiazolidinone compounds (including MMPT and DBPT) were evaluated by cell viability assays. For this purpose, human lung cancer cell lines

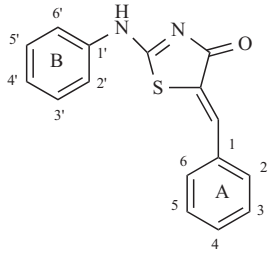

Fig. (1A). Backbone of 5-Benzylidene-2-Phenylimino-1, 3-Thiazolidin-4-one (BPT).

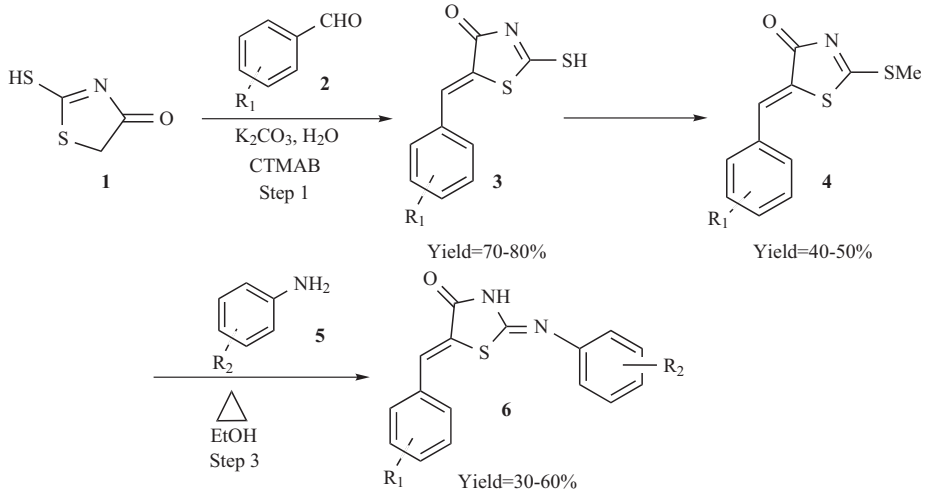

Fig. (1B). Synthetic route to BPT analogs.

Table 1. BPT Analogs Tested

Group No.	Analog Name	Molecule Formula	LogP*	Substituent #	
				Ring A	Ring B
I	MMPT	$C_{17}H_{14}N_2OS$	4.10	4-CH_3	
	DBPT	$C_{16}H_{12}N_2O_3S$	2.83	2-OH 4-OH	
	BPT-3	$C_{18}H_{16}N_2O_3S$	3.43	2-OH 4-OEt	
	BPT-4	$C_{16}H_{12}N_2O_2S$	3.22	3-OH	
	BPT-5	$C_{17}H_{14}N_2O_3S$	3.10	3-OCH_3 4-OH	
	BPT-6	$C_{20}H_{18}N_2O_3S$	4.05	3-OCH_3 4-OCH_2CHCH_2	
	BPT-7	$C_{17}H_{12}N_2O_3S$	3.17	3-COOH	
	BPT-8	$C_{16}H_{12}N_2O_2S$	3.22	4-OH	
	BPT-9	$C_{18}H_{15}N_3O_2S$	2.52	4-NHAc	
	BPT-10	$C_{17}H_{14}N_2O_3S$	3.10	2-OH 3-OCH_3	
	BPT-11	$C_{16}H_{12}N_2O_2S$	3.22	2-OH	
	BPT-12	$C_{18}H_{16}N_2O_3S$	3.36	2-OCH_3 4-OCH_3	
	BPT-13	$C_{17}H_{14}N_2O_2S$	3.93	4-OCH_3	
	BPT-14	$C_{18}H_{16}N_2O_3S$	3.67	3-OCH_3 4-OCH_3	
	BPT-15	$C_{18}H_{16}N_2O_3S$	3.36	2-OCH_3 5-OCH_3	
II	BPT-16	$C_{17}H_{14}N_2O_3S$	2.71	3-OCH_3 4-OH	4'-OH
	BPT-17	$C_{18}H_{16}N_2O_4S$	2.58	3-OCH_3 4-OH 5-OCH_3	4'-OH
	BPT-18	$C_{17}H_{14}N_2OS$	4.10		4'-CH_3
	BPT-19	$C_{17}H_{12}C_{l2}N_2O_2S$	4.60	2-OCH_3	3'-Cl 4'-Cl
	BPT-20	$C_{18}H_{16}N_2O_3S$	3.36	3-OCH_3	4'-OCH_3
	BPT-21	$C_{16}H_{11}ClN_2O_2S$	3.78	4-Cl	4'-OH
	BPT-22	$C_{19}H_{18}N_2O_3S$	3.85	2-OCH_3 3-OCH_3	4'-CH_3
	BPT-23	$C_{18}H_{16}N_2O_4S$	2.97	2-OCH_3 5-OCH_3	4'-OH
	BPT-24	$C_{19}H_{18}N_2O_4S$	3.23	2-OCH_3 5-OCH_3	4'-OCH_3

(Table 1. Contd….)

Group No.	Analog Name	Molecule Formula	LogP*	Substituent #	
				Ring A	Ring B
	BPT-25	$C_{18}H_{16}N_2O_4S$	2.97	2-OCH_3 4-OCH_3	4'-OH
	BPT-26	$C_{19}H_{18}N_2O_4S$	3.23	2-OCH_3 4-OCH_3	4'-OCH_3
	BPT-27	$C_{18}H_{17}N_3O_3S$	2.56	2-OCH_3 4-OCH_3	4'-NH_2

* LogP was calculated using Crippen's fragmentation Method

The numbers indicate the positions where substitution occurs as in Fig.(1A).

H460 and H1299, human colon cancer cell lines Lovo and DLD-1, human breast cancer cell line MDA-MB-231, and normal human fibroblasts were seeded in 96-well plates and treated with each compound at various concentrations. The IC_{50} of these compounds obtained by XTT assay are summarized in Table **2**. Seven of the analogs killed none of the six cell lines, at concentrations ranging from 31nM to 31μM. Six of analogs killed some of cancer cell lines or normal cells, but their IC_{50} values were higher than that of MMPT. Seven of the analogs had lower IC_{50} values, indicating that they were more potent than MMPT in inducing cytotoxic effects. Of these eight, four (DBPT, BPT-5, 12, 16 and 27) were less toxic to normal human fibroblast cells than MMPT (Fig. **2**).

By comparing substituents and cytotoxic effects, we found that simple substitution of 4-CH_3 in MMPT by 4-OH, 4-OCH_3, or 4-NHAc abolished the cytotoxic effects of BPT-8, 13, and 9, respectively, even though their logP are lower than MMPT. Substitutions in ring A with 2-OH or 3-COOH abrogated the cytotoxic effects of BPT-11 and 7. In contrast, a substitution with 3-OH (BPT-4) resulted in cytotoxic effect in normal and cancer cells. These results suggest that polarity or hydrophility at 2- and 4- positions of ring A may dramatically influence cytotoxic effects of the compounds.

Interestingly, while either 4-OH or 2-OH (compounds 8 and 11) in ring A is not cytotoxic, the presence of the both (compound DBPT) is cytotoxic. Moreover, compounds with

Table 2. Median Inhibitory Concentrations of BPT Analogs in Six Cell Types

Analog Name BPT-	IC_{50} (μM) ± SD					
	NHFB	DLD1	MDA-MB-231	H460	H1299	LOVO
Group A						
6	>31	>31	>31	>31	>31	>31
7	>31	>31	>31	>31	>31	>31
8	>31	>31	>31	>31	>31	>31
9	>31	>31	>31	>31	>31	>31
11	>31	>31	>31	>31	>31	>31
17	>31	>31	>31	>31	>31	>31
18	>31	>31	>31	>31	>31	>31
Group B						
10	13.4±3.46	6.2±0.14	>31	9.6±1.56	5.2±1.56	2.4±0.21
13	>31	5.2±0.07	>31	26.3±1.69	1.6±0.42	1.9±0.21
14	>31	>31	8.5±0.92	25.7	>31	16.7±4.52
19	22.2±3.35	7.8±0.21	13.4±1.77	>31	13.4±1.48	1.75±0.21
21	>31	>31	>31	>31	20.5±13.4	2.6±0.42
22	16.5±5.51	>31	7.6±0.28	21.4±2.12	>31	6.5±0.98

(Table 2. Contd….)

Analog Name BPT-	IC_{50} (μM) ± SD					
	NHFB	**DLD1**	**MDA-MB-231**	**H460**	**H1299**	**LOVO**
Group C						
MMPT	11.5±2.12	1.9±0.07	2.4±0.70	2.8±0.09	2.0±0.14	10.5±0.70
DBPT	17.5±4.94	0.51±0.35	4.4±0.07	3.4±0.07	1.6±2.10	3.9±3.96
3	5.3±0.42	>31	4.1±0.78	2.6±0.14	3.30±.71	1.35±0.21
4	3.4±0.42	1.9±1.2	3.3±0.42	5.9±0.49	2.3±0.28	2.9±0.35
5	15.8±1.06	7.8±0.78	5.6±0.78	4.1±1.27	4.8±0.42	5.4±1.20
12	18.7±1.77	1.1±0.07	0.83±0.67	2.6±0.35	15.9±3.32	0.44±0.07
16	>31	0.80±0.08	12.3±14.3	2.5±0.42	0.48±0.21	0.73±0.09
20	6.9±2.89	3.50±0.21	0.73±0.15	3.4±0.28	3.5±0.21	1.8±0.07

two substitutive groups consisting of -OH, $-OCH_3$, or both in ring A (DBPT, BPT-3, 5, 10, 12, and 14) have cytotoxic effects for at least some cancer cells. Compound 6 is not active, however, presumably because of an unfavorable substituent size of -OCH2CH=CH_2.

Compounds 16 and 20, which have 4'-OH and 4'-OCH_3 modifications in ring B, have a greater degree of cytotoxicity. Notably, compounds 16 and 5 have the same modifications in ring A. Yet, addition of 4'-OH in ring B increased the cytotoxic effect of compound 16 in four of five cancer cell lines tested. However, a further modification of ring A of compound 16, addition of 5-OCH_3 (compound 17), abolished cytotoxic effects. Moreover, changing the methyl group of MMPT in ring A to position 4 in ring B (analog 18) also abrogated the cytotoxic effect of the compound. It is possible that an increase of polarity or hydrophility in ring B would enhance the cytotoxic effect of the compounds. Thus, hydrophility or polarity of substituents and their positions in both rings may play roles in the cell-killing activity and cell-type specificity of the compounds.

Cell-killing Activity in Chemoresistant Cancer Cells

Our recent study [12-13] showed that both MMPT and DBPT are active in P-glycoprotein over-expressing cells. To test whether the compounds in Table **2** that induced tumor-selective cytotoxic effects were also effective for chemoresistant cells, we determined the cytotoxic effects of MMPT, DBPT, and BPT-12 and 16 in parental H460 cells and their paclitaxel- or vinorelbine-resistant derivatives, H460/TaxR and H460/VinR. We found that all four compounds induced dose-dependent cell killing in all three cell lines (Fig. **3**). Interestingly, IC_{50} and IC_{90} values were comparable for those compounds in parental H460 cells; the IC_{50} and IC_{90} values for the resistant cells were markedly lower for compound R (Table **3**). This result demonstrates that analogs of BPT can induce cytotoxic effects like those of MMPT in P-glycoprotein-over- expressing cells.

Uptake of Compounds by Cancer Cells

The P-glycoprotein-independent cell killing induced by some BPT analogs suggests that those compounds are not

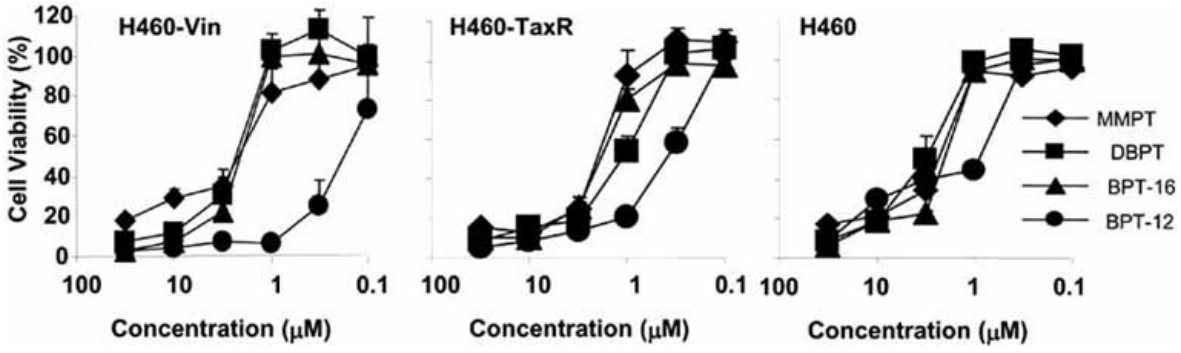

Fig. (2). Cell viability in H460, H460/VinR, and H460/TaxR cells. Cells were treated with MMPT, DBPT, analog BPT-12, or analog BPT-16 at concentrations indicated. Cell viability was determined by SRB assay 4 d after treatment. Cells treated with the same amount of DMSO (v/v: 0.1%) were used as controls, whose viability was set 1. The values represent mean ± SD of a quadruplicate assay.

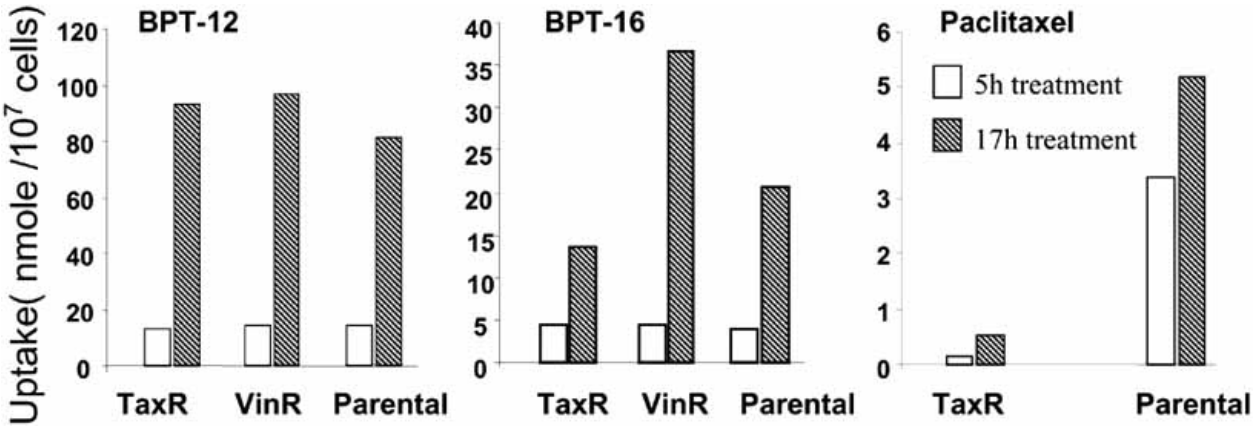

Fig. (3). Uptake of compounds by cells. Cells were incubated with 10 μg/ml of analog BPT-12 or analog BPT-16, or 8 μg/ml of paclitaxel, for 5 or 17 h in cell culture medium. After rigorous washing, compounds remaining in cells were extracted and quantified by HPLC analysis. The data represent one of two experiments with similar results.

substrates of P-glycoprotein. To test whether intracellular accumulation of the compounds is affected by P-glycoprotein status, we determined levels of compounds inside of cancer cells after treatment with the compound. For this purpose, we treated H460, H460/TaxR, and H460/VinR cells with 10μg/ml concentrations of analogs BPT-12 or 16. Cells were harvested 5 or 17 h later and compounds inside of cells were extracted as described in Materials and Methods. The extract was then subjected to HPLC and the amount of each compound within 1×10^7 cells was determined through normalization with an internal standard as described in Materials and Methods. Analog BPT-9 was used as the internal standard compound. As shown in Fig. (**4**) analog 9 can be separated from 12 and 16 completely (resolution >1) with HPLC, and the signal response factors for BPT-12 and 16 were 1.011 and 1.425, respectively.

The results show that uptake of BPT-12 and 16 in parental H460, H460/TaxR, and H460/VinR cells increased over time from 5 h to 17 h (Table **4**). Overall, the uptake of BPT-12 in these three cancer cell lines was much better than that of analogue 16, although the water-solubility of BPT-16 (logP=2.536) is higher than that of BPT-12 (logP=3.882) based on their logP values. Nevertheless, the results showed that accumulation of these compounds in the cancer cells was not affected by level of drug efflux pump P-glycoprotein. In contrast, the intracellular accumulation of paclitaxel was dramatically lower in H460/TaxR cells than in parental H460 cells. This result suggests that BPT-12 and 16 are not substrates of P-glycoprotein.

In summary, we obtained 27 analogs of BPT and analyzed 21 analogs for their cytotoxic effects in cancer and normal cells. The results showed that the cell killing effects of these compounds were affected by modifications occurring in two phenyl rings of BPT. We also found that, in addition to MMPT and DBPT, compounds 5, 12 and 16 induced tumor-selective cytotoxicity, when compared to their effects on normal human fibroblasts. Moreover, compound 12 was more effective than these other analogs in killing cancer cells that are resistant to paclitaxel or vinorelbine and overexpress P-glycoprotein. Analysis of cell uptake of these compounds demonstrated that accumulation of these compounds in cancer cells was not affected by P-glycoprotein. Together, the tumor-selective cytotoxic effects and effectiveness in cancer cells overexpressing P-glycoprotein indicate that these compounds might be useful for treatment of refractory tumors. Nevertheless, clinical application of these agents may also depend on their *in vivo* efficacy and toxicity, and their chemical and pharmacological properties, including absorption, distribution, metabolism, and excretion [16-19]. Thus, much work remains to be done to determine whether some BPT analogs can be used for treatment of cancers.

Table 3. Inhibitory Concentrations of BPT Analogs in Sensitive and Resistant Cancer Cells

MMPT and Analog	IC_{50}/ IC_{90} (μM)			
	H460/TaxR	H460/VinR	H460	NHFB
MMPT	2.0/>31	1.1/>31	2.8/>31	11.5/>31
DBPT	1.1/17	2.5/16	3.4/20	17.5/>31
BPT-12	0.36/5.9	0.15/0.79	2.6/25	18.7/>31
BPT-16	1.9/14	2.2/6.8	2.5/18	>31

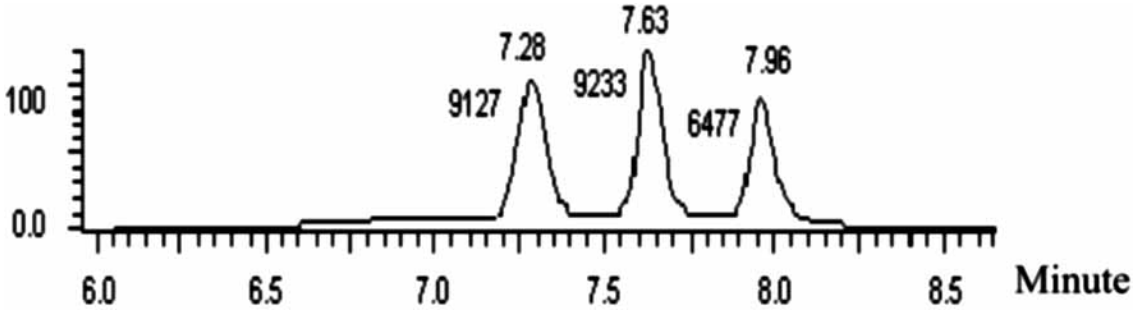

Fig. (4). Chromatography of Analogs BPT-9, 12, and 16. Analog BPT-9 was used as the internal standard compound. It can be separated from BPT-12 and 16 completely (resolution >1) with HPLC, Retain Time (min): BPT-16=7.28, BPT-9=7.63, BPT-12=7.96. Signal Response (Area): BPT-16 =9127, BPT-9 =9233, BPT-12 =6477 and the signal response factors for BPT-16 and BPT-12 were 1.011 and 1.425, respectively.

MATERIALS AND METHODS

Chemicals

Twenty one analogs of BPT were purchased from Chem Bridge Corporation. They were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co., St. Louis, MO) as stock solution (5mg/ml). Unless otherwise stated, DMSO was used as the control in all studies.

Preparation of BPT Analogs in Small Scales

The BPT analogs were synthesized by 3 steps (Fig. **5**). In the first step [20], rhodanine (1) reacted with substituted benzylaldehyde (2) in water media at ambient temperature for 12-24 hrs to obtain substituted 5-benzylidene rhodanine (3) precipitate (yield 70-80%). Methylation [21] of substituted 5-benzylidene rhodanine was carried out in step 2 with MeI in EtOH under catalysis of $(iPr)_2NEt$ for 1-4 hr (yield 40-50%). In the last step, heating was applied in reaction between 5-benzylidene-2-(methylthio)thiazolidin-4-one (4) and substituted benzylamine (5). The reaction was carried out at 78-80˚C for 0.5 - 4 hr, depending on the substituents in the ring A & B (yield 30-60%). The crude products were purified by silica gel column chromatography. Eluents of $CHCl_3$/MeOH or $CHCl_3$/Aceton/MeOH was used for elution of compounds from silica gel column. The purity and molecule weight of the final product were determined by high performance liquid chromatography-mass spectrum (LC/MS). To further confirm their chemical identity, we also determined the NMR spectrum of some BPT analogs. NMR analysis was performed at Nuclear Magnetic Resonance Facility of our institution, using a Bruker 500 MHz DRX NMR Spectrometer with Me_4Si as an internal standard**.** For example, the purity and mass for BPT-12 synthesized in house and analyzed by LC-MS were 96% and 341, respectively. The BPT-12 obtained commercially and BPT-12 synthesized in house have the same NMR profile. An example of NMR data for BPT-12 is as following:

^{1}H NMR (500 MHz, CDCl3) δ 8.68 (s, 1H), δ 7.42 (t, 2H, J=7.87 Hz), δ 7.33 (d, 1H, J=8.75 Hz), δ 7.23 (t, 1H, J=7.87 Hz), δ 7.11 (d, 2H, J=7.87 Hz), δ 5.63 (dd, 1H, J1=8.75 Hz, J2=2.62 Hz), δ 6.47 (d, 1H, J=2.62 Hz), δ 3.88 (s, 3H), δ 3.86 (s, 3H); The singlet at 8.68 was very broad possibly due to tautomarization. ^{13}C NMR (125 MHz, CDCl3) δ (162.7, 159.8, 130.3, 129.3 (2Cs), 127.2, 125.1, 121.6 (2Cs), 121.5, 115.9, 105.3, 98.4, 55.5, 55.5 doublet), two carbons on the hetero cycle were not observable possibly due to long relaxation times.

Cell Lines and Cell Culture

Human lung cancer cell lines H1299 and H460, human breast cancer cell line MDA-MB-231, and human colon adenocarcinoma cell lines LoVo and DLD-1 were maintained in

Table 4. Uptake of BPT Analogs 12 and 15 in H460 Cancer Cells

Cancer Cell Line	Treatment Duration (h)	Concentration in Cell (n mole/10^7 cells)		
		BPT-12	BPT-16	Paclitaxel
H460/TaxR	5	12.9	4.4	0.135
	17	93.2	13.7	0.522
H460/VinR	5	14.4	4.4	
	17	96.5	36.5	
H460	5	14.4	3.8	3.39
	17	80.9	20.6	5.19

our laboratory. Paclitaxel-resistant H460/TaxR and vinorelbine-resistant H460/VinR cells were derived from H460 and overexpress P-glycoprotein as reported previously [12]. Normal human fibroblasts (NHFB) were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 1X medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% antibiotics and cultured at 37°C in a humidified incubator where atmosphere contained 5% CO_2.

Cell Viability Analysis

Cell viability was determined by 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazoliumhydroxide (XTT) assay as described previously [14-15]. Briefly, 1 day after 4000 cells/well in 100μl medium were cultured in 96-well plates, the cells were treated with BPT analogs at concentrations ranging from 31 nM to 31μM. DMSO alone was used as a control. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Four days after treatment, the medium was removed and cells were washed once with phosphate-buffer saline (PBS). Cell viability was measured by XTT assay using Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. Each experiment was performed in quadruplicate and repeated at least twice. Cell viability was calculated according to this formula: $A_{treatment}/A_{control}$ x 100 (%). Concentrations that inhibited 50% or 90% of cell growth (IC_{50} and IC_{90} respectively) were determined by using Curve Fit Software.

Extraction of BPT Analogs in Cancer Cells

To determine the uptake of BPT analogs by cancer cells, cancer cells (1×10^7) in dishes (φ=15 cm) were treated with them at a concentration of 10 μg/ml concentration for 5 or 17 h. Cells were then washed three times with PBS to remove free compounds, harvested, and counted. To release compounds from the cells, cells were spun at 1200 rpm for 2 min, and the resulting cell pellets were then suspended in 1-1.5 ml PBS and subjected to three freeze/thaw cycles. Adding H_3PO_4 to a final concentration of 0.02% disrupted the compound-protein interaction/binding. After incubation at room temperature for 10 min, cell lysates were spun at 13,500 rpm for 10 min at 4ºC. The supernatants with compounds were collected. Protein precipitates were washed with 0.5-1 ml PBS and the supernatant was pooled together. BPT analogs in the pooled supernatant were extracted by the solid phase extraction method using the Oasis HLB (C-18) 1cc (30 mg) cartridge (Waters Corp., Milford, MA) and following the products instruction. Before adding sample to the cartridge, it was conditioned with 1 ml of methanol and then 1 ml of distilled water. After sample all passed through the cartridge, the cartridge was washed with 2 ml of distilled water. The compound remaining in the cartridge was extracted by elution with 2 ml of methanol.

Analytical High Performance Liquid Chromatography

Chromatographic analysis of compounds in cell extracts was performed by using the HP1100 binary pump HPLC inlet system (Agilent Technologies, Wilmington, DE) and a Phenomenex Luna 5μ C5 50 x 2.0 mm column (Phenomena, Torrance, CA). The sample was separated by application of the rapid linear gradient of 40% to 100% methanol in 3.5 min. The mobile phase consisted of 0.1% formic acid water solution as phase A and methanol as phase B. A flow rate of 300 μl/ml was employed. The column was maintained at 40°C.

To quantitatively analyze the BPT analogs in cancer cell with HPLC, BPT-9 was chosen as the internal standard (Istd), and was added to 2 ml of the extractants already described in same amount. Fresh analogs 12 and 15 were employed as standards (Std) to determination of the response factor (RF) by HPLC according to the following equation:

Response Factor = (IStd peak area/Std peak area) × (Std amount/ IStd amount)

The contents of analogs G and R in sample were calculated by this equation:

Amount of analyte = RF × IStd amount × peak area of analyte/peak area of IStd

ACKNOWLEDGMENTS

We thank Kathryn Hale for editorial review. This work was supported in part by National Cancer Institute grants CA 092487-01A1 and CA 098582-01A1 (to B.F.), Lung SPORE (CA 70907) development award, and Cancer Center Core grant CA16672.

REFERENCES

[1] Advani, R.; Fisher, G. A.; Lum, B. L.; Hausdorff, J.; Halsey, J.; Litchman, M.; Sikic, B. I. *Clin. Cancer Res*. **2001**, *7*, 1221-1229.
[2] Ambudkar, S. V.; Kimchi-Sarfaty, C.; Sauna, Z. E.; Gottesman, M. M. *Oncogene* **2003**, *22,* 7468-7485.
[3] Cucco, C.; Calabretta, B. *Cancer Res.* **1996**, *56,* 4332-4337.
[4] Ferry, D. R.; Traunecker, H.; Kerr, D. J. *Eur. J. Cancer* **1996**, *32A,* 1070-1081.
[5] Fojo, A. T.; Ueda, K.; Slamon, D. J.; Poplack, D. G.; Gottesman, M. M.; Pastan, I. *Proc. Natl. Acad. Sci. USA* **1987**, *84,* 265-269.
[6] Gottesman, M. M.; Fojo, T.; Bates, S. E. *Nat. Rev. Cancer* **2002**, *2*, 48-58.
[7] Gu, J.; Kagawa, S.; Takakura, M.; Kyo, S.; Inoue, M.; Roth, J. A.; Fang, B. *Cancer Res.* **2000**, *60,* 5359-5364.
[8] Huesker, M.; Folmer, Y.; Schneider, M.; Fulda, C.; Blum, H. E.; Hafkemeyer, P. *Hepatology* **2002**, *36*, 874-884.
[9] Kartner, N.; Riordan, J. R.; Ling, V. *Science* **1983**, *221,* 1285-1288.
[10] Kuznetsova, M.; Fokina, A.; Lukin, M.; Repkova, M.; Venyaminova, A. *Nucleosides, Nucleotides Nucleic Acids* **2003**, *22,* 1521-1523.
[11] Lin, J. H.; Lu, A. Y. *Pharm. Rev.* **1997**, *49,* 403-449.
[12] Lipinski, C. A. J. *Pharmcol. Toxicol. Meth.* **2000**, *44,* 235-249.
[13] Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery. Rev.* **2001**, *46*, 3-26.
[14] Prentis, R. A.; Lis, Y.; Walker, S. R. *Brit. J. Clin. Pharmacol.* **1988**, *25,* 387-396.
[15] Samuels, B. L.; Hollis, D. R.; Rosner, G. L.; Trump, D. L.; Shapiro, C. L.; Vogelzang, N. J.; Schilsky, R. L. *Clin Cancer Res.* **1997**, *3,* 1977-1984.
[16] Teraishi, F.; Wu, S.; Sasaki, J.; Zhang, L.; Zhu, H.; Davis, J. J.; Fang, B. *J. Pharmacol. Exp. Ther.* **2005**, *314,* 355-362.
[17] Teraishi, F.; Wu, S.; Zhang, L.; Guo, W.; Davis, J. J.; Dong, F.; Fang, B. *Cancer Res.* **2005**, *65,* 6380-6387.

[18] Wu, H.; Hait, W. N.; Yang, J. M. *Cancer Res.* **2003**, *63,* 1515-1519.
[19] Zhang, L.; Gu, J.; Lin, T.; Huang, X.; Roth, J. A.; Fang, B. *Gene Ther.* **2002**, *9,* 1262-1270.
[20] Wang, Y.; Ren, Z.; Cao, W.; Tong, W. *Shanghai Daxue Xuebao, Ziran Kexueban* ***2004****, 10*, 79-81.
[21] Song, Y.; Connor,D.; Doubleday, R.; Sorenson, R. J.; Sercel, A. D.; Unangst, P. C.; Roth B. D.; Gilbertsen, R. B.; Chan, K.; Schrier, D. J.; Guglietta, A.; Bornemeier, D. A.; Dyer, R. D. *J. Med. Chem.* ***1999****, 42,* 1151-1160.

Received: 06 February, 2006 **Revised: 23 June, 2006** **Accepted: 24 June, 2006**