

Original Research Article

Combined Effect of Vorinostat and Grape Seed Proanthocyanidins on Modulation of Thymidine Phosphorylase in Non-Small Cell Lung Cancer

Yuan-Yuan Wu^{1*}, Ting-Ting Cao² and Chun-Ling Liu¹

¹Department of Medical Oncology, ²Department of Ophthalmology, Cangzhou Central Hospital, Cangzhou 061001, China

*For correspondence: **Email:** yuanyuanwu167@gmail.com; **Tel/Fax:** 0086-317-2079783

Received: 12 February 2015

Revised accepted: 25 May 2015

Abstract

Purpose: To demonstrate the effect of histone deacetylase-inhibitor, vorinostat, on antitumour activity of grape seed proanthocyanidins (GSPs) in non-small cell lung cancer (NSCLC) cells.

Methods: Expression of thymidine phosphorylase (TP) and thymidylate synthase (TS) was measured by real-time PCR and western blotting. TP knockdown was performed using specific small interfering RNA. Antitumour activity of combination of vorinostat and GSPs was assessed according to Chou and Talay method and by evaluating apoptosis.

Results: Vorinostat treatment led to a significant increase in TP expression but decrease in TS expression in NSCLC cells. In H157 cells, increase in the concentration of vorinostat from 0.34 to 0.4 μ M increased TP expression 3- to 6-fold. In H1299 cells, there was 7-fold reduction of TS transcript and 30-fold increase of TP transcript at 48 h. Vorinostat, when used in combination with GSPs, resulted in a synergistic anti-proliferative effect and increased apoptotic cell death. However, cells with TP knockdown did not exhibit vorinostat- and GSPs-mediated anti-proliferative effect and apoptotic cell death.

Conclusion: The combination of vorinostat and GSPs can be an effective and innovative antitumour therapy for the treatment of NSCLC.

Keywords: Histone deacetylase-inhibitor, Synergism, Apoptosis, Antitumor

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Grape seed proanthocyanidins (GSP), mixture of polyphenols and flavanols exhibit anticancer activity with least toxicity [1-3]. The major portion of the extract comprises proanthocyanidins (89 %) like catechins and/or (-)-epicatechins [1,3]. For determination of chemotherapeutic effects of GSPs an extract of grape seeds is used. It is reported that dietary GSPs lead to inhibition of lung tumor cell proliferation and the inhibition of angiogenic factors in human NSCLC cell xenografts in nude mice [4].

The anticancer agents, histone deacetylase inhibitors (HDAC-Is), regulate expression, survival and differentiation of cell cycle genes in tumour cells without affecting normal tissues [5-8]. There are several HDAC-Is in preclinical models [6], whereas some are in advanced clinical development either individually or in combination with conventional chemotherapeutics or targeted agents [6,8]. It is reported that expression of 2-5 % of the genes is selectively altered by histone deacetylase inhibitors [6]. Vorinostat (suberoylanilide hydroxamic acid), the most promising HDAC-I exhibited promising preclinical and clinical

antitumour activity. It is the first HDAC-I approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma in patients with progressive, persistent or recurrent disease during or following two systemic therapies [7]. HDAC-I are also believed to play a vital role in thymidylate synthase (TS) expression [9-13]. Thymidylate synthase has a vital role in the synthesis of thymidilate (dTMP) and subsequently DNA synthesis [14]. It is reported that overexpression of TS induces resistance to 5-FU in most of the tumours [14]. Recently it was reported that vorinostat induces inhibition of TS protein in tumour cells [12].

Taking into consideration the promising antitumour effect of vorinostat we devised a study to investigate the effect of vorinostat and GSPs in NSCLC cells. In the present study, we provided evidence that by down regulating TS and up regulating TP mRNA and protein expression, vorinostat demonstrated synergistic antitumour activity in combination with GSPs.

EXPERIMENTAL

Materials and cell culture

Vorinostat, GSPs, tubacin and niltubacin were purchased from Sigma-Aldrich, USA. Human A549, H1299, and H157 NSCLC cell lines were purchased from American Type Culture Collections (Rockville, MD, USA). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated foetal bovine serum, 50U mL^{-1} of penicillin, 500 mgmL^{-1} of streptomycin, and 4 mmolL^{-1} of glutamine in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

Cell survival/proliferation assay

To 96-well flat bottom multiplates (BD Falcon, Franklin, NJ) containing 1×10^5 cells per well various concentrations of the drugs were added. After incubation for 96 h, sulforhodamine B (ICN Biomedicals, Irvine, CA, USA) was added to each well and the plates were again incubated for 4 h at 37 °C in 5 % CO_2 incubator. A microplate reader (MPR-A4i; Tosoh Corporation, Tokyo, Japan) was used to measure absorption at 570 nm. All the measurements were carried out in triplicate.

In vitro drug combination studies

To determine drug combination, a range of equipotent doses of the two compounds were tested in combination. The plot of viable cells against concentration of the compounds tested

was used obtain combination index (CI) using Chou–Talalay equation [12,15-18,]. For synergistic, additive or antagonistic effects the value of combination index corresponds to < 0.9, 0.9 – 1.2 and > 1.2, respectively. The dose reduction index (DRI) for each compound was also evaluated.

Western blot analysis

The cells after treatment were lysed, and using dye-binding method (Bio-Rad) protein concentration was determined. The proteins were resolved on 15 % SDS-PAGE and transferred to nitrocellulose membranes. Digitonin-based subcellular fractionation technique was used for cytosolic and mitochondrial fractions. Onto SDS-PAGE equal volumes of cytosolic and mitochondrial fractions were resolved and transferred to nitrocellulose membranes. The membranes after incubation with primary antibody were washed, and then incubated with horseradish peroxidase anti-mouse or horseradish peroxidase anti-rabbit antibodies. Enhanced chemiluminescence system was used for visualization of immunoreactive bands. The primary antibodies used were: thymidylate synthase (TS), acetyl-H3, poly-(ADP-ribose)-polymerase (PARP), g-tubulin, platelet-derived endothelial growth factor (TP) and GAPDH, cleaved caspase 3 and BAX-antibodies from Cell Signalling Technology (Boston, MA, USA).

Analysis of apoptotic cell death by flow cytometry

For determination of apoptosis of the human non-small cancer cells flow cytometry using the Annexin V–conjugated Alexa Fluor488 (Alexa488) Apoptosis Detection kit was employed. The cells after overnight serum starvation were treated with different concentrations of vorinostat and/or 50-DFUR for 96 h. The cells were harvested and washed in PBS. Then the cells were incubated with Alexa488 and propidium iodide in the dark. FACS Calibur instrument (BD Biosciences) equipped with the Cell Quest 3.3 software was used for analysis of stained cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was used for reverse transcription using DNA synthesis kit (Invitrogen). Primers for PCR were

designed and PCR amplification of cDNA was performed at 35 cycles in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01 % (w/v) gelatin, 200 μM dNTP, TS and TP-specific primers (0.5 μM each), and 2.0 U of platinum *Taq* DNA polymerase (*Invitrogen*). For each reaction, two negative controls were performed consisting of omission of the RT step or omission of the target cDNA.

Transient knockdown of TP using siRNA

A siRNA sequences against human TP and a negative control Duplexes used as control for non-sequence-specific effects were obtained from (*Invitrogen*). Thymidine phosphorylase siRNA or control siRNA (33 nM) was transiently transfected into the cells using Lipofectamine 2000 (*Invitrogen*). Briefly, siRNA: Lipofectamine mixture complexes were incubated for 20 min at room temperature and then added to cells growing in petri dish. After 48 h the complexes were replaced by complete medium and cells were treated as indicated and assayed.

Statistical analysis

All the results are expressed as mean ± standard deviation (SD, n = 3). Statistical significance of differences was determined by two-sided Student's t-test and one-way ANOVA. Differences were considered statistically significant at $p < 0.05$. Sigma Stat software (Systat Software Inc., San Jose, California, USA) was used for all statistical evaluations.

RESULTS

Antiproliferative effects of vorinostat and GSPs in NSCLC cell lines

All the three (A549, H1299, and H157) NSCLC cell lines exhibited similar cytotoxicity to vorinostat. Two out of three cell lines (A549 and H1299) were more sensitive to GSPs compared to H157 cells (Table 1).

Table 1: Sensitivity of NSCLC cancer cell lines to vorinostat and GSPs

Cell lines	IC ₅₀ (± SD) Vorinostat (mM)	IC ₅₀ (± SD) GSPs (mM)
A549	0.8 ± 0.7	0.8 ± 0.2
H1299	0.9 ± 0.8	0.6 ± 0.1
H157	0.5 ± 0.2	1.6 ± 0.3

Effect of vorinostat on TS and TP expression in NSCLC cell lines

The results from western blot analysis revealed a significant decrease in TS protein expression and increase in TP protein expression on treatment with vorinostat for 48 h in A549, H1299, and H157 cell lines. The observed effect was concentration dependent and was significant from the concentration of 0.34 μM vorinostat (Figure 1A). In H157 cells, the increase in concentration of vorinostat from 0.34 to 0.4 μM increased the TP expression from 3 to 6-fold.

Vorinostat-induced down regulation of TS lead to clear down regulation of TS transcript in A549 cells after 12 h with a four-fold reduction after 48 h. Similar results were obtained for H1299 cells (Figure 1B). The vorinostat-induced up regulation of TP enhanced TP transcript in A549 cells that was also evident after 12 h with a 27-fold induction at 48 h. (Figure 1D). In H1299 cells, there was 7-fold reduction of TS transcript at 48 h and 30-fold increase of TP transcript at 24 h (Figure 1C,E).

Synergistic antitumour effects of vorinostat in combination with GSPs

Determination of the CIs values (< 0.8) for the antitumour effect of vorinostat and GSPs combination in A549, H1299, and H157 cells revealed synergistic anti-proliferative effects. The synergistic effect was observed on treatment with equipotent doses of vorinostat and GSPs for 48 h at 50 % (CI₅₀) or 75 % (CI₇₅) of cell lethality (Table 2). There was a dose reduction in IC₅₀ values (DRI₅₀) to 6-fold for both vorinostat and GSPs in combination compared with the concentrations of the two drugs alone.

The results from flow cytometry showed a significant increase in apoptotic cell death of A549 and H1299 cells by combination therapy compared with individual treatments after 96 h treatment (Figure 2A,B). The doses used were IC₃₀ values of vorinostat (0.34 and 0.20 μM, respectively) and of GSPs (3 and 5 μM, respectively) in A549 and H1299 cells for 96 h. The expression of the proapoptotic protein BAX and the cleavage of PARP were enhanced in both A549 and H1299 cells on treatment with vorinostat and GSPs combination (Figure 2C,D).

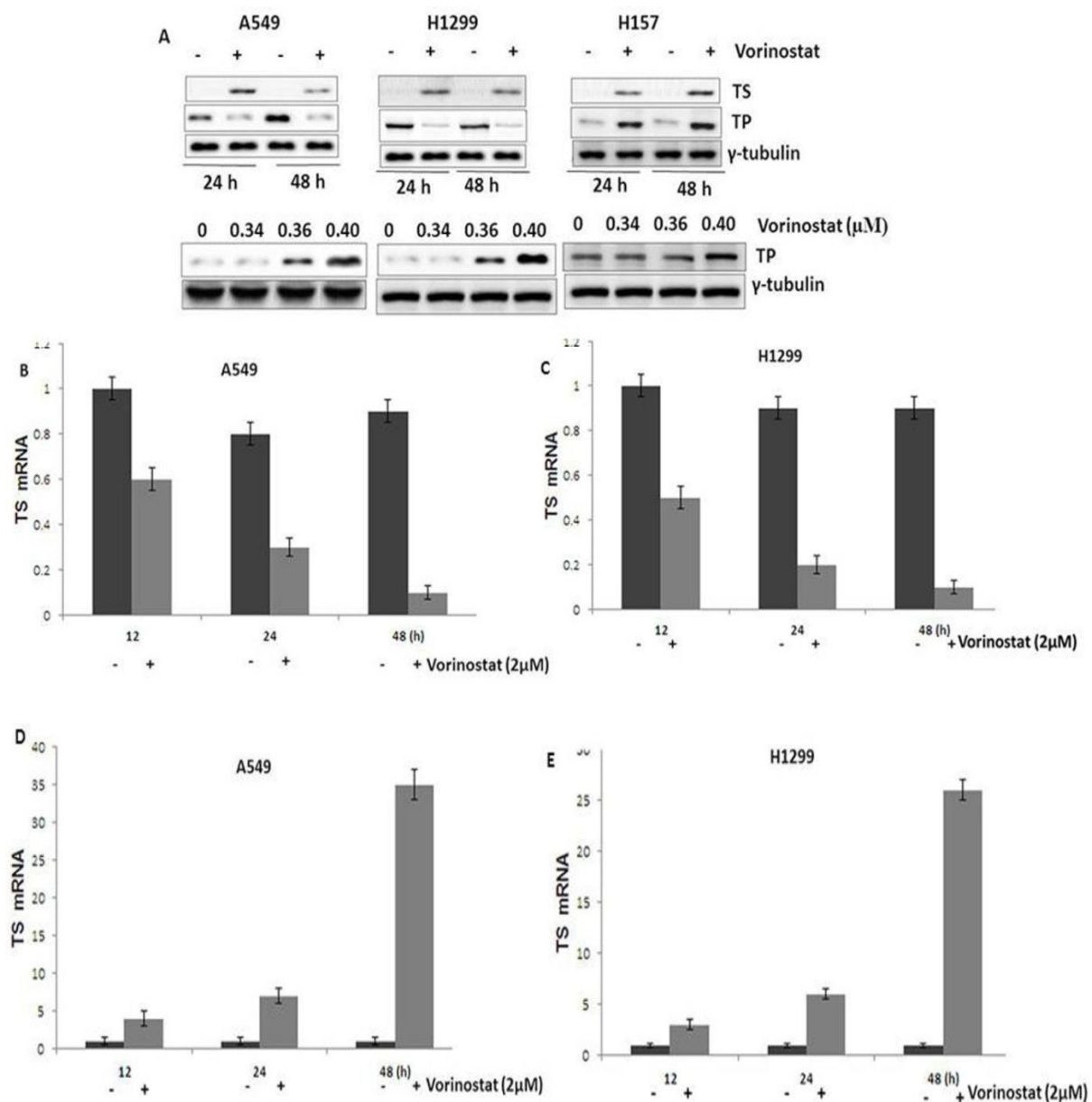


Figure 1: Effect of vorinostat on TS and TP expression in NSCLC cells. (A) TS and TP proteins expression were determined by western blotting on the indicated A549 and H1299 cells untreated or treated with vorinostat at concentration corresponding to IC₅₀ 48 h. γ-Tubulin was used as protein loading control. (B-E) TS and TP mRNA expression were determined by real-time PCR

Table 2: Combination index (CI) and dose reduction index (DRI) values for vorinostat and GSPs combination treatment

Cell line	^a CI ₅₀ ± SD vorinostat + GSPs	CI ₇₅ ± SD vorinostat + GSPs	^a DRI at IC ₅₀ ± SD	
			Vorinostat	GSPs
A549	0.86 ± 0.27	0.69 ± 0.21	2.7 ± 0.89	5.7 ± 1.23
H1299	0.73 ± 0.29	0.58 ± 0.18	2.1 ± 0.92	4.8 ± 1.01
H157	0.89 ± 0.28	0.47 ± 0.13	2.96 ± 1.12	4.6 ± 0.95

^aFor CI and DRI values mean ± s.d. from at least three separate experiments performed in quadruplicates were computed at 50 and 75 % of cell death (CI₅₀ and CI₇₅, respectively) according by CalcuSyn software after 96 h of treatment

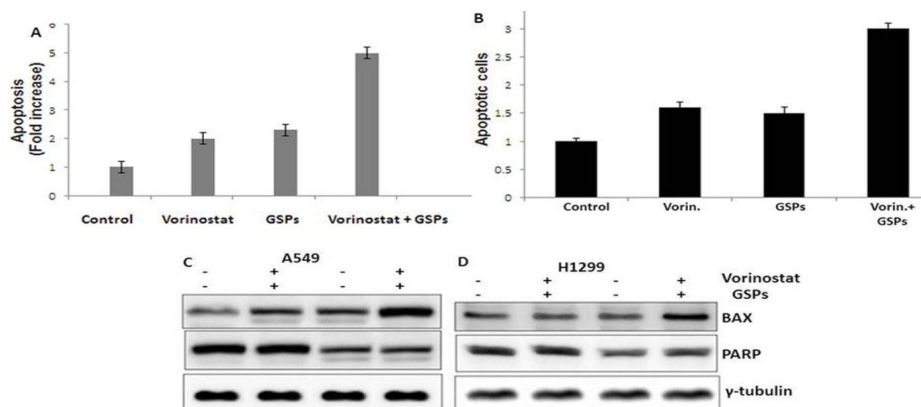


Figure 2: Apoptotic effect of vorinostat in combination with GSPs on NSCLC cells. Apoptosis was evaluated by flow cytometry analysis after nuclear DNA staining with propidium iodide in A549 (A) and H1299 (B) cells untreated or treated for 72 h with vorinostat (at 0.34 and 0.20 μM , respectively) and/or GSPs (at 3 and 5 μM , respectively). Western blot analysis of BAX and PARP were performed on A549 (C) and H1299 (D) cells untreated or treated for 72 h with vorinostat (0.35 and 0.20 μM , respectively) and/or GSPs (3 and 5 μM , respectively); γ -tubulin was used as protein loading control

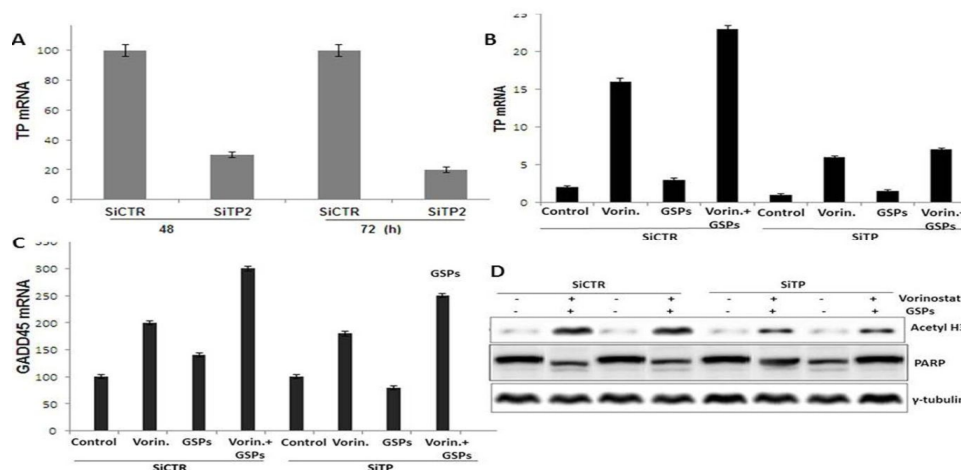


Figure 3: Knockdown of TP reduces the apoptotic effect of vorinostat in combination with GSPs on NSCLC cells. (A) Small interfering RNA (siRNA) for TP (siTP) significantly reduced TP mRNA expression determined by real-time PCR both after 48 and 72 h from transfection compared with control siRNA cells (siCTR). siCTR and siTP NSCLC cells, 24 h after transfection were untreated or treated for 24 h with vorinostat and/or GSPs and TP (B) and GADD45 (C) mRNA expression were determined by real-time PCR. (D) Western blot analysis of PARP and acetyl histone H3 were performed on siCTR and siTP NSCLC treated as in B and C. γ -Tubulin was used as protein loading control

Knockdown of TP reduces the apoptotic effect of vorinostat in combination with GSPs

For investigation of the relation of TP expression and observed synergistic effect of vorinostat/GSPs on apoptosis, a specific siRNA was used to knockdown TP. The results showed a significant decrease in TP mRNA levels in the A549 cells transfected with siTP construct compared to control siRNA transfected cells 72 h from transfection (Figure 3A). TP knocked down also leads to reduction in the efficiency of vorinostat to enhance TP expression compared

with control cells (Figure 3B). There was also no effect of vorinostat/GSPs combination on the GADD45, a growth-arrest and DNA-damage-inducible gene in the cells with TP knocked down compared to that of control cells (Figure 3C). In addition, the cells with TP knocked down did not show enhanced cleavage of PARP and caspase 3 on treatment with vorinostat/GSPs combination as observed in control cells (Figure 3D). Thus suggesting the importance of vorinostat induced TP expression up regulation for the synergistic anti-proliferative and apoptotic cell death induced by vorinostat/GSPs combination.

DISCUSSION

The results of the present study revealed a synergistic interaction between vorinostat and GSPs in NSCLC. We showed that the anti-proliferative effect induced by vorinostat in NSCLC cells was mediated through decreased TS protein expression, the vital enzyme involved in the synthesis of thymidilate, and by increase in the expression of TP protein. Our results showing vorinostat-induced TS protein expression down-regulation via TS mRNA transcript are in agreement with earlier reports [9-13]. Our results for the first time revealed synergistic antitumour interaction between vorinostat and GSPs in a preclinical model. Earlier it was reported that promoter methylation can lead to inhibition of TP expression in human cancer cells. We also demonstrated that knockdown of TP by specific siRNA significantly inhibits the apoptotic cell death induced by synergistic interaction of vorinostat/GSPs combination.

It is reported that TP-inducible chemotherapeutics including taxanes, cisplatin or cyclophosphamide, in combination with capecitabine increased response rate, time to progression and survival in breast cancer patients in phase III studies. The phase II studies of gastric and NSCLC have also shown promising results using the above combinations [19-22]. The maximum concentration of vorinostat tolerable by human body was determined using two phase I pharmacokinetic and pharmacodynamics studies in combination with FOLFOX or 5-FU/LV chemotherapy regimens [23,24] in CRC.

CONCLUSION

The results indicate that vorinostat has the unique capability to modulate not only TS but also TP expression in tumour cells and, consequently, can synergise with GSPs. In addition, both vorinostat and GSPs are drugs that can be administered orally with consequent increased compliance by patients. Thus, the combination of vorinostat and GSPs is a promising chemotherapeutic strategy for the treatment of SCLC cells.

REFERENCES

1. Nandakumar V, Singh T, Katiyar SK. Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Lett* 2008; 269: 378-87.
2. Sharma SD, Meeran SM, Katiyar SK. Dietary grape seed proanthocyanidins inhibit UVB-induced oxidative

stress and activation of mitogen-activated protein kinases and nuclear factor- κ B signaling in *in vivo* SKH-1 hairless mice. *Mol Cancer Ther* 2007; 6: 995-1005.

3. Mittal A, Elmets CA, Katiyar SK. Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. *Carcinogenesis* 2003; 24: 1379-88.
4. Akhtar S, Meeran SM, Katiyar N, Katiyar SK. Grape seed proanthocyanidins inhibit the growth of human non-small cell lung cancer xenografts by targeting insulin-like growth factor binding protein-3, tumor cell proliferation, and angiogenic factors. *Clin Cancer Res* 2009; 15: 821-31.
5. Budillon A, Di Gennaro E, Bruzzese F, Rocco M, Manzo G, Caraglia M. Histone deacetylase inhibitors: a new wave of molecular targeted anticancer agents. *Recent Pat Anticancer Drug Discov* 2007; 2: 119-134.
6. Budillon A, Bruzzese F, Di Gennaro E, Caraglia M. Multiple-target drugs: inhibitors of heat shock protein 90 and of histone deacetylase. *Curr Drug Targets* 2005; 6: 337-351.
7. Marks PA. Discovery and development of SAHA as an anticancer agent. *Oncogene* 2007; 26: 1351-1356.
8. Lane AA, Chabner BA. Histone deacetylase inhibitors in cancer therapy. *J Clin Oncol* 2009; 27: 5459-5468.
9. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol Cancer Ther* 2003; 2: 151-163.
10. Lee JH, Park JH, Jung Y, Kim JH, Jong HS, Kim TY, Bang YJ. Histone deacetylase inhibitor enhances 5-fluorouracil cytotoxicity by downregulating thymidilate synthase in human cancer cells. *Mol Cancer Ther* 2006; 5: 3085-3095.
11. Tumber A, Collins LS, Petersen KD, Thouggaard A, Christiansen SJ, Dejligbjerg M, Jensen PB, Sehested M, Ritchie JW. The histone deacetylase inhibitor PXD101 synergises with 5-fluorouracil to inhibit colon cancer cell growth *in vitro* and *in vivo*. *Cancer Chemother Pharmacol* 2007; 60: 275-283.
12. Di Gennaro E, Bruzzese F, Pepe S, Leone A, Delrio P, Subbarayan PR, Avallone A, Budillon A. Modulation of thymidilate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5FU or raltitrexed. *Cancer Biol Ther* 2009; 8: 782-791.
13. Fazzino W, Wilson PM, Labonte MJ, Lenz HJ, Ladner RD. Histone deacetylase inhibitors suppress thymidilate synthase gene expression and synergize with the fluoropyrimidines in colon cancer cells. *Int J Cancer* 2009; 125: 463-473.
14. Ackland SP, Clarke SJ, Beale P, Peters GJ. Thymidilate synthase inhibitors. *Cancer Chemother Biol Response Modif* 2002; 20: 1-36.

15. Magne N, Fischel JL, Dubreuil A, Formento P, Marcie S, Lagrange JL, Milano G. Sequence-dependent effects of ZD1839 ('Iressa') in combination with cytotoxic treatment in human head and neck cancer. *Br J Cancer* 2002; 86: 819-827.
16. Bruzzese F, Rocco M, Castelli S, Di Gennaro E, Desideri A, Budillon A. Synergistic antitumor effect between vorinostat and topotecan in small cell lung cancer cells is mediated by generation of reactive oxygen species and DNA damage-induced apoptosis. *Mol Cancer Ther* 2009; 8: 3075-3087.
17. Bruzzese F, Di Gennaro E, Avallone A, Pepe S, Arra C, Caraglia M, Tagliaferri P, Budillon A. Synergistic antitumor activity of epidermal growth factor receptor tyrosine kinase inhibitor gefitinib and IFN-alpha in head and neck cancer cells in vitro and in vivo. *Clin Cancer Res* 2006; 12: 617-625.
18. Avallone A, Di Gennaro E, Bruzzese F, Laus G, Delrio P, Caraglia M, Pepe S, Comella P, Budillon A. Synergistic antitumor effect of raltitrexed and 5-fluorouracil plus folinic acid combination in human cancer cells. *Anticancer Drugs* 2007; 18: 781-791.
19. Kindwall-Keller T, Otterson GA, Young D, Neki A, Criswell T, Nuovo G, Soong R, Diasio R, Villalona-Calero MA. Phase II evaluation of docetaxel-modulated capecitabine in previously treated patients with non-small cell lung cancer. *Clin Cancer Res* 2005; 11: 1870-1876.
20. Walko CM, Lindley C. Capecitabine: a review. *Clin Ther* 2005; 27: 23-44.
21. Ajani J. Review of capecitabine as oral treatment of gastric, gastroesophageal, and esophageal cancers. *Cancer* 2006; 107: 221-231.
22. Gelmon K, Chan A, Harbeck N. The role of capecitabine in first-line treatment for patients with metastatic breast cancer. *Oncologist* 2006; 11: 42-51.
23. Fakhri MG, Fetterly G, Egorin MJ, Muindi JR, Espinoza-Delgado I, Zwiebel JA, Litwin A, Holleran JL, Wang K, Diasio RB. A phase I study of two schedules of vorinostat in combination with 5-fluorouracil and leucovorin in patients with refractory solid tumors. *Clin Cancer Res* 2010; 16: 3786-3794.
24. Fakhri MG, Pendyala L, Fetterly G, Toth K, Zwiebel JA, Espinoza-Delgado I, Litwin A, Rustum YM, Ross ME, Holleran JL, Egorin MJ. A phase I, pharmacokinetic and pharmacodynamic study on vorinostat in combination with 5-fluorouracil, leucovorin, and oxaliplatin in patients with refractory colorectal cancer. *Clin Cancer Res* 2009; 15: 3189-3195.