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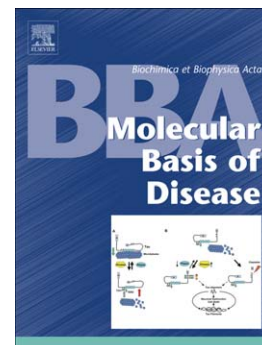
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A TYROSINE KINASE INHIBITOR, β -HYDROXYISOVALERYLSHIKONIN, INDUCED APOPTOSIS IN HUMAN LUNG CANCER DMS114 CELLS THROUGH REDUCTION OF dUTP NUCLEOTIDOHYDROLASE ACTIVITY

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Abbreviations: β -HIVS, β -hydroxyisovalerylshikonin; PTK, protein tyrosine kinase; PLK1, polo-like kinase 1; TRAP1, tumor necrosis factor receptor-associated protein 1; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; dUTPase, dUTP nucleotidohydrolase; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; 5-FU, 5-fluorouracil; DTT, dithiothreitol; NAC, N-acetyl-cysteine; MS, mass spectrometry.

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Abstract

Apoptotic cell death was induced in human lung cancer DMS114 cells by treatment with β -hydroxyisovalerylshikonin (β -HIVS), an ATP-noncompetitive inhibitor of protein tyrosine kinases. Changes in phosphoprotein profiles were analyzed by two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE) after the cells were treated with β -HIVS. One spot on the 2D gel showed a marked decrease in intensity and the corresponding protein was identified by mass spectrometry as dUTP nucleotidohydrolase (dUTPase). The β -HIVS-induced decrease of dUTPase in the phosphoprotein fraction of DMS114 cells was confirmed using immunoblotting. Treatment of the cells with β -HIVS induced rapid reduction of dUTPase activity. An antioxidant *N*-acetyl-cysteine inhibited both the reduction of phosphorylated dUTPase and the induction of apoptosis by β -HIVS treatment of DMS114 cells. Introduction of siRNA directed against dUTPase mRNA into DMS114 cells enhanced the susceptibility of β -HIVS-induced apoptosis. Treatment of DMS114 cells with β -HIVS and 5-fluorouracil, a specific inhibitor of thymidylate synthase used as a chemotherapeutic drug, revealed the synergistic effects of these drugs on the inhibition of cell growth. These results suggest that dUTPase activity is one of the crucial factors involved in apoptotic cell death in lung cancer cells.

(184 words)

1. Introduction

Lung cancer is one of the leading cause of death in developed countries and the number of patients has been growing in Japan [1,2]. Metastasis and drug resistance are the major causes of mortality in lung cancer, and the therapeutic results await further improvement. Induction of apoptotic cell death specifically in cancer cells would be an ideal way to combat tumors, because apoptotic cell death dose not induce an inflammatory response. We have searched for various agents that induce apoptotic cell death preferentially in cancer cells. Geranylgeraniol induced apoptosis in U937 leukemia cells through enhanced release of cytochrome *c* from mitochondria [3]. Vitamin K₂ was found to be active apoptosis inducer on ovarian cancer cells [4].

5-fluorouracil (5-FU) is a well known anti cancer drug currently used for several tumors including lung cancer. 5-FU is an inhibitor of thymidylate synthetase, which catalyzes the conversion of dUMP to thymidylate, which is required for the synthesis of DNA. One of the enzymes that provides dUMP, the substrate of thymidylate synthase, is dUTP nucleotidohydrolase (dUTPase). dUTPase is a ubiquitous enzyme that hydrolyzes dUTP to yield dUMP and pyrophosphate. Recent studies have demonstrated that inhibition of thymidylate synthase causes accumulation of dUTP, which leads to misincorporation of uracil into DNA and results in lethal damage to DNA [5]. dUTPase is a major regulator of the size of the dUTP pool and it protects cells from DNA damage by limiting the accumulation of dUTP. Thus, activation of the expression of the gene for dUTPase results in resistance to inhibitors of thymidylate synthase, such as 5-FU [6,7], fluorodeoxyuridine [8], and ZD9331 [9].

We previously reported that β -hydroxyisovalerylshikonin (β -HIVS), which is isolated from the roots of the traditional oriental medicinal herb *Lithospermum radix*, can inhibit the growth of various lines of human cancer cells at concentrations between 10^{-8} and 10^{-6} M [10]. Among the cancer cells examined, β -HIVS was able to induce cell death the most efficiently in DMS114 and NCI-H522 lung cancer cells [11]. It is interesting that β -HIVS is an ATP-noncompetitive inhibitor of tyrosine kinases such as v-Src and EGFR [11]. Indeed β -HIVS induces apoptosis in DMS114 cells via a protein tyrosine kinase (PTK)-dependent pathway [12].

It is well known that protein phosphorylation is the major signaling machinery in various cellular phenomena, such as cell cycle, cellular locomotion, and apoptosis. Several protein kinases are reported to be involved in the regulation of apoptosis in cancer cells [13-15]. Many of the studies, including our previous one, utilize inhibitors of protein kinases to show the potential involvement of certain protein phosphorylation in apoptosis, however such an

approach might not be suitable for elucidating the downstream mechanisms that induce final events. In the present study, we used another strategy to clarify details of the mechanism in induction of apoptosis in cancer cells by β -HIVS. Since most cell-death and cell-proliferation signals are transmitted via phosphorylation and dephosphorylation of proteins, we focused on changes in the phosphorylation of proteins during induction of apoptosis in cancer cells by β -HIVS. Phosphoproteins in DMS114 cells were fractionated using a phosphoprotein-purification column and analyzed these phosphoproteins by two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE). We found that the intensity of one spot on the 2D gel decreased significantly after treatment of cells with β -HIVS and we identified the corresponding protein by mass spectrometry as dUTPase. Ladner *et al.* found both mitochondrial and nuclear isoforms of dUTPase in human cells [16,17]. Ladner *et al.* indicated that the nuclear isoform of dUTPase is a phosphoprotein and Ser11 is the only phosphorylated site *in vivo* [18]. They also demonstrated that neither the enzymatic activity nor intracellular localization of dUTPase is affected by the phosphorylation [18, 19]. Since β -HIVS is a specific inhibitor of tyrosine kinase, its direct effect on dUTPase is not expected and its effect may be transmitted through signaling cascade.

2. Materials and methods

2.1. Cell Culture and Reagents

We cultured DMS114 human small lung carcinoma cells in RPMI 1640 medium (GIBCO, Glasgow, UK) supplemented with 10% heat-inactivated fetal bovine serum. β -HIVS was isolated from the plant *Lithospermum radix* as described previously and dissolved in ethanol [10]. Phosphoproteins were fractionated with a PhosphoProtein Purification kit (QIAGEN Inc., Darmstadt, Germany). Polyclonal dUTPase-specific antibodies were kindly provided by Dr. Robert D. Landner of the University of Medicine and Dentistry of New Jersey, USA. The equipment for 2D-PAGE, Multiphor II, and Dry Gel Strips (pH 4-7) were purchased from Amersham Sciences (Piscataway, NJ, USA). HPLC-grade water, formic acid, and acetonitrile were from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Proteomics grade trypsin was obtained from Promega (Madison, WI, USA). 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and olomoucine were purchased from Sigma (St Louis, MO, USA).

2.2. Preparation of Phosphoprotein

Phosphoproteins were enriched with the PhosphoProtein Purification kit [20]. In brief, DMS114 cells were washed with 20 mM TBS buffer (20 mM Tris-HCl, pH 7.6, 0.15 M NaCl) and lysed with phosphoprotein lysis buffer that contained 0.25 % CHAPS, Benzonase and protease inhibitors (QIAGEN Inc., Darmstadt, Germany). After incubation for 30 min at 4 °C, samples were centrifuged at 13,000 g at 4 °C for 30 min. Then 2.5 mg of protein in the lysate were applied on phosphoprotein-purification column. The flow-through fraction was collected and then, after the column had been washed with lysis buffer that contained 0.25% CHAPS, 500 µl of phosphoprotein-elution buffer, containing 0.25% CHAPS, was applied and the eluate was collected. Each sample was desalted with the VIVAspin system (Sartorius, Hannover, Germany) and a 2-D Clean-Up Kit (Amesham Biosciences Corp., Piscataway, NJ, USA).

2.3. High-Resolution Two-dimensional Gel Electrophoresis

Proteins were separated, according to the manufacturer's instructions, on an immobilized pH gradient-based isoelectric dry gel in the first dimension and by SDS-PAGE in the second dimension. We applied 200 µg of protein to each 7-cm isoelectric dry gel overnight, and then the gel was allowed to equilibrate in 50 mM Tris-HCl buffer (pH 8.8) that contained 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue. Electrophoresis of the first dimension was conducted according to the manufacturer's instructions. Electrophoresis in the second dimension was conducted in a slab gel that contained 12% acrylamide and 0.1% SDS in Laemmli's buffer system [21]. The proteins were detected by silver staining. Image analysis and database management were performed with Melanie 3 image-analysis software from GeneBio (Geneva, Switzerland).

2.4. In-gel Reduction, Alkylation, and Digestion of Proteins

For preparation of samples for analysis by micro-mass (micro-MS) spectrometry, the silver-stained spots of protein were cut from the gel, washed in water, immersed in acetonitrile, and dried with a centrifuge-concentrator (TOMY SEIKO Co. Ltd., Tokyo, Japan). Dried fragments of the gel were reduced by heating at 56°C for 30 min in 100 mM dithiothreitol (DTT) that contained 100 mM NH₄HCO₃, and alkylated by incubation at 37°C for 30 min in darkness with 100 mM iodoacetamide in 100 mM NH₄HCO₃. The fragments of gel were then washed in 100 mM NH₄HCO₃, incubated in acetonitrile and dried in the centrifuge-concentrator. Then each fragment was rehydrated for 45 min on ice in a solution of 1.25 µg of sequencing-grade trypsin

(Promega, Madison, WI) in 5 μ l of 10 mM Tris-HCl (pH 8.8). Each sample was digested at 37°C overnight. Then each supernatant was removed and collected in a fresh tube. Each fragment was then extracted twice with 5% formic acid in 50% acetonitrile. All supernatants from a given fragment were combined and concentrated to near dryness in the centrifuge-concentrator. Each concentrated sample was mixed with 0.1% trifluoroacetic acid and applied to a ZipTipC₁₈ column (Millipore, Billerica, MA, USA). After the columns had been washed with 0.1% trifluoroacetic acid, the sample was eluted with 75% acetonitrile. Then the acetonitrile was removed with the centrifuge-concentrator.

2.5. Immunoblotting Analysis

After samples had been prepared with the PhosphoProtein Purification kit, they were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was rinsed with TBST [20 mM Tris (pH 7.4), 0.15 M NaCl, 0.05% Tween 20] and then blocked with 5% skim milk in TBST for 1 h at room temperature. The blocked membrane was subsequently probed for 1 h at room temperature with diluted (1:1000) polyclonal antibodies against dUTPase. After the membrane had been washed three times with TBST, it was incubated for 1 h with horseradish peroxidase-conjugated goat antibodies against rabbit IgG. Bands of immunoreactive proteins were detected with an ECL Kit (PerkinElmer Life Sciences, Inc., Boston, MA, USA).

2.6. Detection of the Induction of Apoptosis

Apoptotic cells were detected by staining of cells with Hoechst 33342 (Sigma, St. Louis, MO, USA). Hoechst 33342-staining was conducted at room temperature after treatment of cells with β -HIVS. Triplicate samples of a minimum of 200 cells each were counted under fluorescence microscope (BXSO; Olympus, Tokyo, Japan) and apoptotic cells were identified as cells with condensed and fragmented nuclei as described previously [22]. Apoptosis was also evaluated by measuring the formation of mono- and oligonucleosomes using Cell-Death detection ELISA^{PLUS} kit (Boehringer Mannheim, Mannheim, Germany) in accordance with the manufacturer's instructions. The fragmentation of nucleosomes released into the cytosolic fraction was expressed in terms of the difference in absorption at 405 nm and 495 nm.

2.7. Quantification of Cell Proliferation

Cell proliferation was quantified in terms of absorbance at 492 nm after exposure of cells to 2,3-bis(2-methoxy-4-nitro-5-sulfohenyl)-2H-tetrazolium-5-carboxanilide (XTT). In brief, DMS114 cells (10^4 cells in 100 μ l of medium per well) were treated with β -HIVS for 24 h. After treatment, 50 μ l of a solution of XTT (1 mg/ml) and phenazine methosulfate (PMS; 0.383 mg/ml) were added. Culture was continued for a further 4 h and then absorbance was measured at 492 nm with a microplate reader (Toyo Soda Kogyo, Ltd., Tokyo, Japan).

2.8. dUTPase-specific siRNA Preparation and Introduction into Cells

siRNA that corresponded to part of the sequence of dUTPase mRNA was designed with a 5' phosphate, a 3' hydroxyl group, and a two-base overhang (TT) at the 3' end of each strand was synthesized by QIAGEN Inc. The following sequences were used to target dUTPase mRNA: sense strand, 5'- AGGUGAUCGAAUUGCACCAGd(TT)-3'; and antisense strand, 5'-d(TT) UCCACUAGCUUAACGUGUC-3' (nucleotides 404-424; Genebank accession No. BC033645). The sequence of non-silencing strands are as follows: sense strand, 5'- UUCUCCGAACGUGUCACGUd(TT)-3'; antisense stand, 5'- ACGUGACACGUUCGGAGAAAd(TT)-3'. Complementary strands were annealed by incubation at 90°C for 1 min and then at 37°C for 1 h. DMS114 cells were incubated for 24 h in RPMI 1640 supplemented with 10% fetal calf serum, and then they were treated with 5 μ g of the siRNA in the same culture medium in the presence of RNAiFect transfection reagent (QIAGEN Inc.). After incubation for 48 h, the incubation medium was replaced by fresh RPMI 1640 supplemented with 10% fetal calf serum and then the cells were treated with β -HIVS for 24 h prior to assays.

2.9. Mass-spectrometric Analysis and Identification of Peptides

Peptide mass analysis was performed on a Q-TOF micro-mass spectrometer (Waters, Manchester, United Kingdom). Data were acquired and processed with MassLynx version 4.0 (Nihon Waters, K. K., Tokyo, Japan). Data were submitted to the SWISS-PROT and MSDB databases using MASCOT (MATRIX SCIENCE, Boston, MA, USA) as the search engine and ion scores calculated by MASCOT data base software are used to evaluate the probability of the identification of the peptides [23].

2.10. Measurement of dUTPase Activity

dUTPase activity was measured using a slight modification of the method described by Williams *et al.* [24,25]. After treatment of DMS114 cells with β -HIVS, cells were lysed with phosphoprotein lysis buffer, as described above. The reaction was allowed to proceed at 37°C for 10 min using cell lysate that contained 5 μ g of protein in a total volume of 100 μ l of 50 mM Tris-HCl (pH 7.5) that contained 4 mM MgCl₂, 2 mM β -mercaptoethanol, 0.1% bovine serum albumin, 25 mM NaF, 0.1 mM [5-³H]dUTP (100 μ Ci / μ mol). After the reaction was stopped by heating at 100°C for 3 min, 20 μ l of a mixture of dUTP and dUMP (100 mM each) was added and the reaction mixture was centrifuged at 7,700 x g for 5 min. Ten μ l of the resultant supernatant thus obtained were spotted on a Polygram CEL 300 PEI/UV₂₅₄ plate (Machery-Nagel, Eaton, PA, USA). The plate was developed with 0.5 M LiCl in 2 N acetic acid. The spot corresponding to dUMP was scraped off from the plate and radioactivity was measured in a liquid scintillation counter.

3. Results

3.1. Analysis of Fluctuations in Levels of Proteins during β -HIVS-induced apoptosis in DMS114 cells

As reported previously [22], β -HIVS induces apoptosis in human lung cancer DMS114 cells. Namely, treatment of DMS114 cells with β -HIVS caused release of cytochrome *c* from mitochondria, cleavage and fragmentation of DNA, and condensation and fragmentation of nuclei [22]. When DMS114 cells were treated with 5 μ M β -HIVS and then stained with Hoechst 33342, approximately 35% of the cells displayed morphological changes characteristic of apoptosis, such as condensed and fragmented nuclei (data not shown). To identify proteins that might play important roles in the signal transduction pathways in β -HIVS-induced apoptosis in DMS114 cells, we analyzed phosphoprotein fraction by 2D-PAGE after fractionation of cell lysates on a phosphoprotein-purification column. After DMS114 cells had been treated with 5 μ M β -HIVS for 24 h, the cells were lysed and the lysate was applied to the phosphoprotein-purification column. Phosphoprotein fraction was eluted as described in "MATERIALS AND METHODS". Figure 1B shows typical 2D profiles of eluates derived from DMS114 cells that had been treated with or without β -HIVS. We observed that the intensity of a spot in the phosphoprotein fraction, which had been eluted from the phosphoprotein-purification column, (indicated by the red arrows in Fig. 1B) was reproducibly reduced by treatment of cells with β -HIVS. By contrast, the intensity of the spot due to a well-known phosphoprotein, stathmin [26], indicated by the blue arrows in Figure 1B, was unchanged by treatment of DMS114 cells with β -HIVS. Using the spot of stathmin as a

landmark, we estimated that intensity of the spot indicated by the red arrows had decreased by more than 50%. Under our experimental conditions, we failed to detect any proteins fluctuated reproducibly by the β -HIVS-treatment in the flow-through fraction from the lysate (Fig. 1C). As shown in Figure 1A, a spot corresponding to dUTPase in the lysate fraction could not be detected by silver staining (the red arrows indicate positions of absent spot of dUTPase). These results indicated the phosphoprotein-purification column concentrated the dUTPase.

We cut out the spot indicated by the red arrow in Figure 1B from the gel and digested the material with trypsin after reduction with DTT and alkylation with iodoacetamide, as described in "MATERIALS AND METHODS". After tryptic digestion, we extracted peptides with 50% acetonitrile that contained 5% formic acid and analyzed them by mass spectrometry. As shown in Figure 2A and B, the peptide sequences that we determined matched three partial amino-acid sequences of dUTPase, which is known to be a phosphorylated protein [18].

To confirm that the level of phosphorylated dUTPase in DMS114 cells was reduced by treatment of cells with β -HIVS, we immunoblotted the proteins in the lysate as well as in the eluate and flow-through fractions obtained from the phosphoprotein-purification column (Fig. 2C). The intensity of the band due to dUTPase in the lysate was faint but decreased by approximately 16% upon the treatment with β -HIVS, as estimated by densitometric scanning of the Western blotting bands. As is evident from Figure 2C, the band of dUTPase in the phosphoprotein fraction was markedly reduced after treatment of cells with β -HIVS. No band due to dUTPase in the flow-through fraction was detected.

To validate the above suggestion, we introduced siRNA directed against dUTPase mRNA into DMS114 cells. The nucleotide sequence of dUTPase-specific siRNA used in the present study was different from those used previously [27,28]. As shown in Figure 3A, this siRNA caused a marked decrease in the level of dUTPase itself, as determined by immunoblotting. The expression of TRAP1, which is another molecule that transmits the signal that originates upon exposure of cells to β -HIVS [22], was unaffected by transfection with siRNA against dUTPase (Fig. 3A). Therefore, we used DMS114 cells that contained the siRNA against dUTPase mRNA in a series of experiments in which we examined the effects of the treatment of cells with β -HIVS on the induction of apoptosis. As shown in Figure 3B, dUTPase-specific siRNA-harboring DMS114 cells exhibited slight but significant increase in the extent of induction of apoptosis as compared with that induced by non-silencing siRNA. Neither treatment with RNAiFect alone nor treatment with a control non-silencing siRNA caused any significant increase in the percentage of apoptotic cells. The effect of β -HIVS on the specific siRNA-transfected cells with 2.5 μ M β -HIVS resulted in the markedly enhanced induction of apoptosis, as compared with its effect on the cells that harbored the non-silencing control siRNA (Fig. 3B).

These results indicated that DMS114 cells became more sensitive to β -HIVS upon introduction of siRNA directed against dUTPase mRNA.

3.2. Effects of β -HIVS on dUTPase Activity

Since the level of dUTPase in the phosphoprotein fraction from DMS114 cells was reduced by treatment of cells with β -HIVS, we examined whether the activity of dUTPase might also be changed upon treatment of cells with β -HIVS. As shown in Figure 4A, the percentage of apoptotic cells, as determined by Hoechst 33258-staining under the fluorescent microscope, was increased by treatment with β -HIVS in a dose-dependent manner. Apoptosis was also evaluated by measuring of mono- and oligonucleosomes released into the cytosolic fraction (Fig. 4A). The number of fragmented nucleosomes also increased with increasing concentrations of β -HIVS in parallel with the percentage of apoptotic cells, as determined microscopically in terms of chromatin condensation and nuclear fragmentation. In parallel with the increase in the induction of apoptosis in DMS114 cells by β -HIVS, dUTPase activity in the cells decreased upon treatment of cells with β -HIVS and the effect was dose-dependent (Fig. 4B). As shown in Figure 4C, the induction of apoptosis in DMS114 cells began within 1 h of the start of treatment with 2.5 μ M β -HIVS. dUTPase activity fell by 44% from the control value within 0.5 h after the start of treatment of DMS114 cells with 2.5 μ M β -HIVS (Fig. 4D), suggesting that a decrease in dUTPase activity might trigger the induction of apoptosis in DMS114 cells. The time course changes in amounts of phosphorylated dUTPase in lysates of DMS114 cells that had been treated with β -HIVS were estimated using the phosphoprotein-purification column, as described in "MATERIALS AND METHODS". The results of Western blotting (Fig. 4E) showed clearly that the level of phosphorylated dUTPase eluted from the phosphoprotein-purification column fell sharply 0.5 h after the start of treatment with β -HIVS. The decreased level of phosphorylated dUTPase 0.5 h after the treatment with β -HIVS was approximately 63% as estimated by densitometric scanning of the Western blotting bands of Fig. 4E. These results suggested that the reduction of dUTPase activity in DMS114 cells by β -HIVS might be caused by the decrease in the level of the phosphorylated form of this enzyme.

3.3 Effects of Olomoucine on the Induction of Apoptosis and on the Level of dUTPase in DMS114 cells

Since nuclear dUTPase is phosphorylated by the phosphorylated cyclin-cdc2 complex [18], we examined the effect of olomoucine, an inhibitor of cdc2 [29,30], on the induction of apoptosis

and the phosphorylation of dUTPase in DMS114 cells. As shown in Figure 5A, apoptosis was sharply induced in DMS114 cells by treatment with 100 μ M olomoucine for 24 h. By the incubation of DMS114 cells with olomoucine for 24 h, the level of dUTPase in the phosphoprotein fraction prepared from the olomoucine-treated cells was markedly reduced (Fig. 5B). These results suggested that inhibition of cdc2 by olomoucine leads to decrease in the level of phosphorylated dUTPase. U0126 (10 μ M), SP600125 (10 μ M), and SB203580 (20 μ M), which are inhibitors for ERK, JNK, and p38-MAP kinases, respectively, did not show any effect on the level of dUTPase in the phosphoprotein fraction of DMS114 cells (results not shown).

3.4. Inhibition of β -HIVS-induced suppression of the expression of dUTPase by antioxidant

We previously demonstrated that the induction of apoptosis in DMS114 cells by β -HIVS was inhibited by *N*-acetyl-cysteine (NAC), which is an inhibitor of the accumulation of reactive oxygen species (ROS), suggesting the involvement of ROS in the induction of apoptosis by β -HIVS [22]. In addition, the decrease in levels of TRAP1 upon treatment of DMS114 cells with β -HIVS was inhibited by NAC, suggesting the involvement of ROS in the regulation of the expression of TRAP1 in mitochondria [22]. Therefore, we examined the effect of NAC on suppression of the level of dUTPase in response to β -HIVS. Although the band due to dUTPase in the lysate obtained from untreated DMS114 cells was faint, its intensity was reduced by 20% upon treatment with β -HIVS as quantified by the densitometry and this reduction was inhibited by the presence of NAC (Fig. 6). The band of dUTPase in the eluate fraction obtained from the lysate of DMS114 cells by the phosphoprotein-purification column was also clearly and markedly reduced by treatment with β -HIVS and again the suppression of the level of dUTPase by β -HIVS was inhibited in the presence of NAC. This observation suggests that the level of dUTPase might be regulated by ROS formed as a result of treatment of DMS114 cells with β -HIVS.

3.5. Effects of Various Anticancer Agents

The effects of various apoptosis-inducing anticancer agents on the level and activity of dUTPase in DMS114 cells were examined. The cells were treated with 2.5 μ M β -HIVS, 50 μ M cisplatin, 1 μ M camptothecin, or 100 μ M VP16 (etoposide) for 24 h. Under these conditions, apoptosis was induced in more than 30% of the cells by each drug (Fig. 7A). However, a marked decrease in dUTPase activity was observed only in DMS114 cells treated with β -HIVS (Fig. 7B). Reflecting with this result, a marked decrease in the level of dUTPase in the

phosphoprotein fraction of DMS114 cells occurred only when the cells had been treated with β -HIVS (Fig. 7C).

3.6. Synergistic Effects of β -HIVS and 5-FU on the Inhibition of Growth of DMS114 Cells

Since an increase in the expression of dUTPase in human lung and ovarian tumor cell lines is known to result in resistance to 5-fluorouracil (5-FU), which is an inhibitor of thymidylate synthase and is widely used as an anticancer drug [31], we examined whether the decrease in dUTPase activity in DMS114 cells that was caused by treatment with β -HIVS might change the sensitivity of the cells to 5-FU. As shown in Figure 8, growth of DMS114 cells was inhibited markedly by as little as 1 μ M β -HIVS. By contrast, DMS114 cells were not very sensitive to 5-FU, and treatment with 250 μ M 5-FU inhibited growth of DMS114 cells by only approximately 20% (Fig. 8). Although treatment of DMS114 cells with 0.5 μ M β -HIVS had no significant inhibitory effect on cell growth, 0.5 μ M β -HIVS in combination with either 100 μ M or 250 μ M 5-FU had a marked synergistic inhibitory effect on cell growth. A synergistic inhibitory effect on the growth of DMS114 cells was also observed after treatment of DMS114 cells with either 1 μ M β -HIVS or 2 μ M β -HIVS combined with 5-FU at either 100 μ M or 250 μ M (Fig. 8).

4. Discussion

A protein tyrosine kinase inhibitor, β -HIVS, is a potent inducer of apoptosis against lung cancer cells. To analyze the mechanism for β -HIVS-induced apoptosis, we have carried out proteomic analysis of phosphorylated proteins to analyze the changes in phosphoprotein profiles with or without β -HIVS treatment. We found one protein that reduced markedly after β -HIVS treatment, and this protein was identified as dUTPase.

dUTPase is a ubiquitous enzyme that hydrolyzes dUTP to yield dUMP which is then converted to dTMP by thymidylate synthase. Thymidylate synthase is an important step of nucleotide synthesis, and accumulation of dUTP by inhibition of thymidylate synthase leads to misincorporation of uracil into DNA and results in lethal damage to DNA [5]. Recently, increasing evidences were independently presented to suggest that dUTPase is associated with induction of apoptosis, although the mechanism of its association with apoptosis is not resolved [32,33,34]. In agreement with our results, dUTPase was identified in 12 proteins that their levels were decreased during induction of apoptosis in human Burkitt lymphoma cell line by treatment with anti-IgM antibody [32]. Down regulation of dUTPase in colon cancer HCT116 cells was also observed during apoptosis by treatment with resveratrol, which is a polyphenol

found in red wine [34]. dUTPase in human amnion epithelial cells was also decreased by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, which is a *N*-nitroso alkylating carcinogen [33].

We found in the present study that approximately 44% of the dUTPase activity was lost within 0.5 h after the treatment of DMS114 cells with 2.5 μ M β -HIVS. This decrease in dUTPase activity in DMS114 cells corresponds to the decrease in the level of dUTPase in the phosphoprotein fraction, which also occurred within 0.5 h of the start of treatment with β -HIVS. In addition, the dUTPase activity in the cells was decreased by β -HIVS in a dose-dependent manner, and the extent of the induction of apoptosis was similarly increased with greater concentrations of β -HIVS.

To prove further the involvement of dUTPase in the β -HIVS-induced apoptosis, siRNA directed against dUTPase mRNA was introduced into DMS114 cells. When the expression of dUTPase protein was strongly inhibited, the extent of induction of apoptosis was significantly increased. Furthermore, treatment of dUTPase-specific siRNA-containing cells with β -HIVS showed a synergistic effect on induction of apoptosis, while non-silencing RNA did not enhance β -HIVS-induced apoptosis. From these results, it is suggested that dUTPase activity is directly involved in apoptosis in DMS114 cells. In addition, it is possible that the suppression of dUTPase activity in the cells may increase susceptibility to β -HIVS-induced apoptosis.

Ladner et al. demonstrated that the phosphorylated Ser11 in dUTPase is located within a context that corresponds to the consensus target sequence of the cyclin-dependent protein kinase cdc2 [18]. dUTPase is indeed phosphorylated by the cyclin B1-cdc2 complex [18]. Phosphorylation of cyclin B1 at the position Ser147 by polo-like kinase 1 (PLK1) leads to the cyclin B1-cdc2 complex formation [35]. The association of cdc2 with cyclin B1 and the phosphorylation of cyclin B1 by PLK1 are required for the translocation of the cyclin B1-cdc2 complex to the nucleus [36]. Apoptosis was induced via suppression of the of PLK1 kinase activity after inhibition of PTK activity in leukemia HL60 and U937 cells by β -HIVS [15]. We also demonstrated in the present study that the level of phosphorylated dUTPase in DMS114 cells decreased by treatment with olomoucine, an inhibitor of cdc2, and this treatment induced apoptosis.

A previous report demonstrated that ROS produced upon inhibition of PTK by β -HIVS caused a decrease in levels of TRAP1 in mitochondria and a resultant release of cytochrome *c* from the mitochondria [22]. In the present study, NAC, a scavenger of ROS, efficiently inhibited both the suppression of the level of dUTPase and induction of apoptosis induced by treatment of DMS114 cells with β -HIVS. These results suggested that ROS produced through inhibition of unknown PTK by β -HIVS might be located upstream of the apoptosis-inducing signaling

pathways (Fig. 9). The two signaling pathways, one dependent on mitochondria and the other through phosphorylated dUTPase, might cooperate to induce efficient apoptosis in DMS114 cells in response to β -HIVS (Fig. 9).

5-Fluorouracil is a specific inhibitor of thymidylate synthase and is one of the most widely used chemotherapeutic drugs for the treatment of head and neck, breast, and gastrointestinal cancers [31]. However, successful treatment with 5-FU is often hampered by the development of resistance to this drug. Ladner *et al.* demonstrated that the expression of dUTPase in colon cancer strongly correlated with resistance to 5-FU therapy [6]. In addition, increased expression of dUTPase in human lung H1299 and ovarian SK-OV-3 carcinoma cell lines resulted in significant resistance to 5FU [7]. Thus, a decrease in the level of dUTPase might contribute to an increase in sensitivity of cancer cells to 5-FU. Koehler and Ladner demonstrated that siRNA directed against dUTPase suppressed the expression of this enzyme and sensitized cancer cell lines to growth inhibition by the thymidylate synthase inhibitor fluorodeoxyuridine [27]. The present study demonstrated a significant synergistic inhibitory effect on cell growth when DMS114 cells were treated with 5-FU and β -HIVS together. It is interesting to point out that treatment of lung cancer with 5-FU and β -HIVS simultaneously might have a good therapeutic potential.

We have previously demonstrated that treatment of DMS114 cells with cisplatin and β -HIVS together synergistically inhibited cell growth and induced apoptosis [12]. Although the antitumor effect of cisplatin is believed to be due to the formation of covalent adducts with DNA [37,38], cisplatin also reacts with the sulfhydryl groups of cysteine residues in proteins [37]. We found that tyrosine kinase activities in DMS114 cells were synergistically inhibited by the combined treatment with β -HIVS and cisplatin, and concluded that the synergistic effects observed for DMS114 cells might be due to inhibition of a tyrosine kinase-dependent pathway [12].

In conclusion, the present study demonstrates a new way to increase the sensitivity of lung cancer DMS114 cells to 5-FU using β -HIVS. Our observations suggest the potential clinical utility of combined treatment with inhibitors of tyrosine kinases and thymidylate synthase.

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Figure legends

Fig. 1. Mapping of phosphoprotein and nonphosphoprotein fractions of lysates of DMS114 cells treated with β -HIVS. After DMS114 cells that had been cultured with or without 5 μ M β -HIVS for 24 h, the cells were washed with TBS buffer and lysed with lysis buffer that contained 0.25% CHAPS. The lysate was applied to a phosphoprotein-purification column and phosphoprotein and nonphosphoprotein fractions were obtained fractionated as described in the text. After desalting, the total lysate (A), the eluate fraction (B) and flow-through fraction (C) (200 μ g of protein each) were fractionated 2-D PAGE and then the gels were stained with silver. Stathmin, indicated by blue arrowheads, was utilized as a landmark protein for comparisons of the intensities of spots of dUTPase. The red arrowheads indicate the spots of dUTPase in the eluate fractions. Areas corresponding to dUTPase in the lysate fraction were circled and indicated also by the red arrowheads.

Fig. 2. Identification of dUTPase by mass spectrometry and Western blotting analysis. A, Amino acid sequences of peptides obtained from the spots indicated by a red arrow in Fig. 1B, as

determined by micro-mass spectroscopy and described in the text. B, The amino acid sequence of dUTPase. The underlined sequences correspond to those of peptides identified by mass spectrometry. Ion scores calculated by MASCOT data base software were used to identify the peptides as described in the text. Multiple matched peptides with ions scores above 40 as well as the resulting total ions score above 100 are sufficient for protein identification [23]. C, Western blotting analysis with polyclonal antibodies against dUTPase. DMS114 cells were treated with 5 μ M β -HIVS for 24 h and then lysed. The lysate was fractionated on a phosphoprotein-purification column as described in the text and each fraction containing 5 μ g of protein was used for SDS-PAGE. The result shown is typical of three experiments that gave similar results.

Fig. 3. Effects of transfection of siRNA against dUTPase on DMS114 cells. siRNA against dUTPase mRNA or non-silencing siRNA was introduced into DMS114 cells using RNAiFect reagent, as described in the text. A, Western blotting analysis of lysates of DMS114 cells, as indicated. B, After incubation for 48 h in medium that contained the siRNA against dUTPase mRNA and RNAiFect reagent, the medium was replaced by RPMI 1640 supplemented with 10% fetal calf serum and cells were treated with β -HIVS for 24 h. Apoptotic cells were identified by staining with Hoechst 33342 and counted. Asterisks indicate that the values are statistically significantly different ($*p < 0.01$; $p^{**} < 0.0002$).

Fig. 4. Effects of treatment of DMS114 cells with β -HIVS on the activity of dUTPase. A, Effects of various concentrations of β -HIVS on the induction of apoptosis in DMS114 cells. DMS114 cells were treated with various concentrations of β -HIVS for 24 h and percentages of apoptotic cells (●) and the fragmentation of nucleosomes (○) were determined as described in the “Materials and Methods”. Apoptotic cells (%) were analyzed by staining with Hoechst 33342 and counted as described in the legend to Fig. 3. The fragmentation of DNA was determined by measuring nucleosomes released into the cytosolic fraction using Cell-Death detection ELISA^{PLUS} kit and expressed in terms of the difference in absorption at 405 nm and 495 nm. B, Effects of treatment of DMS114 cells with various concentrations of β -HIVS on dUTPase activity in cells. After DMS114 cells had been treated with various concentrations of β -HIVS for 24 h, dUTPase activity was measured as described in the text. C, Dependence on time of the induction of apoptosis during treatment of DMS114 cells with β -HIVS. After DMS114 cells had been treated with 5 μ M β -HIVS for various times, apoptotic cells were counted as described above. D, Effects of treatment with β -HIVS on dUTPase activity in DMS114 cells. dUTPase activity was measured after treatment of DMS114 cells with 5 μ M β -HIVS for various times. E,

Western blotting analysis of dUTPase in the phosphoprotein fraction of lysates of DMS114 cells that had been treated with β -HIVS for various times. Western blotting analysis of phosphoprotein fractions was performed as described in the legend to Fig. 2.

Fig. 5. Effects of olomoucine on the induction of apoptosis and on the level of dUTPase in DMS114 cells. A, After DMS114 cells had been treated with 100 μ M olomoucine for 24 h, the extent of induction of apoptosis was measured as described in the legend to Fig. 4. B, Effects of olomoucine on the amount of dUTPase. After DMS114 cells had been treated with 100 μ M olomoucine for 24 h, level of dUTPase in the phosphoprotein fraction were determined by Western blotting as described in the legend to Fig. 2.

Fig. 6. Inhibition of β -HIVS-induced suppression of the expression of dUTPase by N-acetylcysteine (NAC). After DMS114 cells were treated with 5 μ M β -HIVS for 24 h in the presence or absence of 1 mM NAC, the cells were lysed as described in Fig.1. Each lysate was fractionated on a phosphoprotein purification column as shown in Fig. 2. Each fraction was analyzed by SDS-PAGE and subsequent immunoblotting as described in the legend to Fig. 2. The result shown is typical of three experiments that gave similar results.

Fig. 7. Effects of various anticancer agents on the induction of apoptosis and dUTPase in DMS114 cells. DMS114 cells were treated with 2.5 μ M β -HIVS, 50 μ M cisplatin, 1 μ M camptothecin (CPT), or 100 μ M VP16 for 24 h. A, Induction of apoptosis in DMS114 cells was measured as described in the legend to Fig. 2. B, Effects on dUTPase activity, which was measured as described in the legend to Fig. 4. C, Western blotting analysis of dUTPase in the phosphoprotein fraction of lysates of DMS114 cells that had been treated with various anticancer agents. Cell lysates were prepared and phosphoprotein fractions were obtained as described in the legend to Fig. 1. Western blotting analysis of the phosphoprotein fractions was performed as described in the legend to Fig. 2.

Fig. 8. Synergistic inhibitory effects of β -HIVS and 5FU on the growth of DMS114 cells. DMS114 cells were treated for 24 h with various concentrations of either β -HIVS or 5FU alone, and of the two drugs in combination. Cell growth was quantified by the XTT assay, as described in the text. The results shown are means \pm SD (bars) of the results obtained from triplicate samples. Three experiments were performed and the results were reproducible.

Fig. 9. Schematic representation of the effect of β -HIVS on dUTPase in DMS114 cells. The inhibition of unknown tyrosine kinase(s) by β -HIVS results in the induction of ROS. NAC, a scavenger of ROS, efficiently inhibited the β -HIVS-induced suppression of dUTPase as well as induction of apoptosis. The expression and activity of PLK1 is also decreased via inhibition of unknown tyrosine kinase(s) by β -HIVS [15]. Phosphorylation of cyclin B1 in the cyclin B1-cdc2 complex by PLK1 is required for translocation of the complex to the nucleus [35] and the phosphorylated cyclin B1-cdc2 complex phosphorylates dUTPase in the nucleus [18]. Treatment of DMS114 cells with olomoucine, an inhibitor of cdc2, inhibited the phosphorylation of dUTPase leading to its decrease in the phosphorprotein fraction. Combined treatment of DMS114 cells with β -HIVS and 5-FU, a specific inhibitor of thymidylate synthase, induced a synergistic growth-inhibitory effect. White arrows indicate changes induced by treatment of DMS114 cells with β -HIVS. The broken lines indicate unknown mechanisms.

Fig. 1

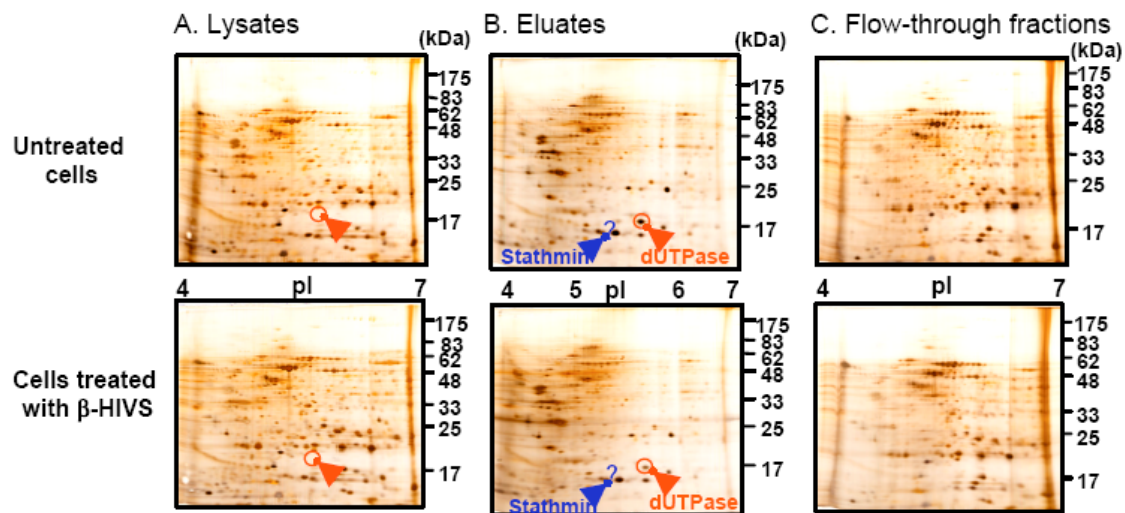


Fig. 2

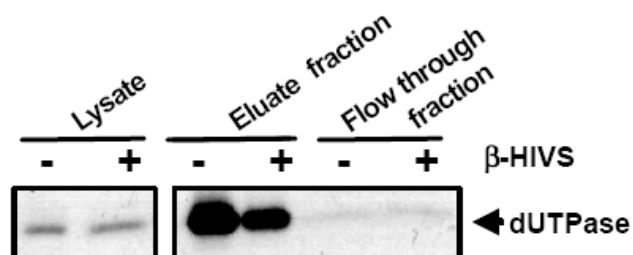
A. Sequences of peptides determined by mass spectrometry

Peptide	Score
LSEHATAPTR	41
IAQLICER + carbamidomethyl (C)	57
GSGGFGSTGKN	51
Total score	150

B. Amino acid sequence of dUTPase

¹⁰ MPCSEETPAI ²⁰ SPSKRARPAE ³⁰ VGGMQLRFAR ⁴⁰ LSEHATAPTR
⁵⁰ GSARAAGLY ⁶⁰ SAYDYTIPPM ⁷⁰ EKAVVKTDIQ ⁸⁰ IALPSGCYGR
⁹⁰ VAPRSLAAK ¹⁰⁰ HFIDVGAGVI ¹¹⁰ DEDYRGNVGV ¹²⁰ VLFNFGKEKF
¹³⁰ EVKKGDRIAQ ¹⁴⁰ LICERIFYPE ¹⁵⁰ IEEVQALDDT ¹⁶⁰ ERGSGGFGST ¹⁷⁰ GKN

C.



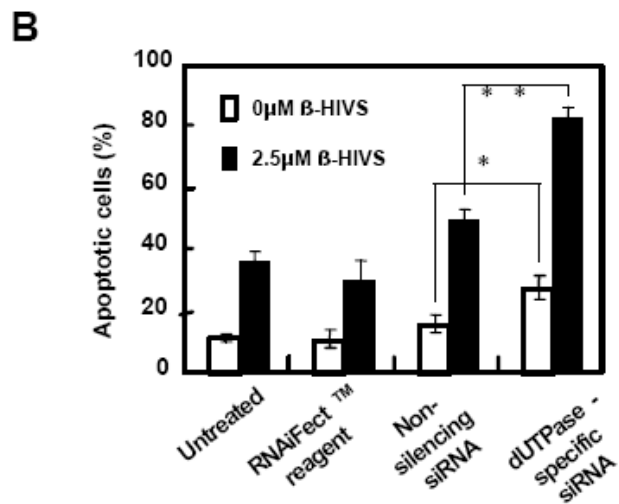
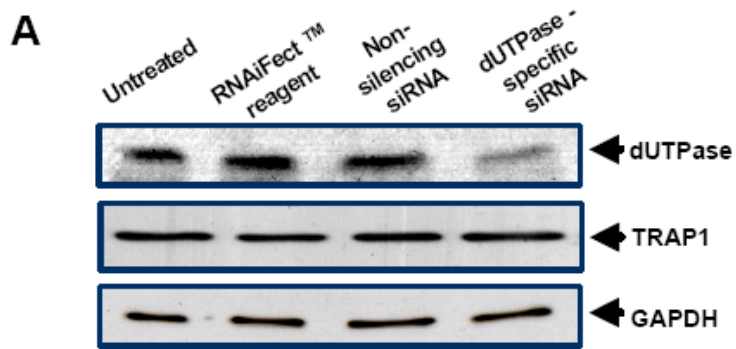


Fig. 3A and 3B

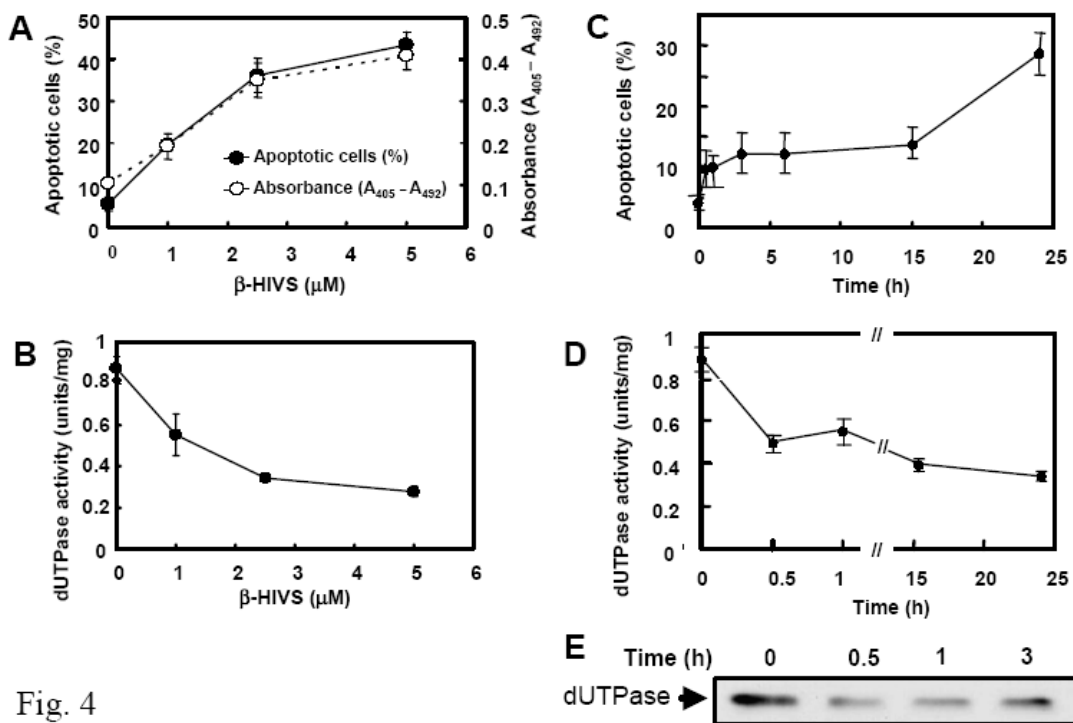
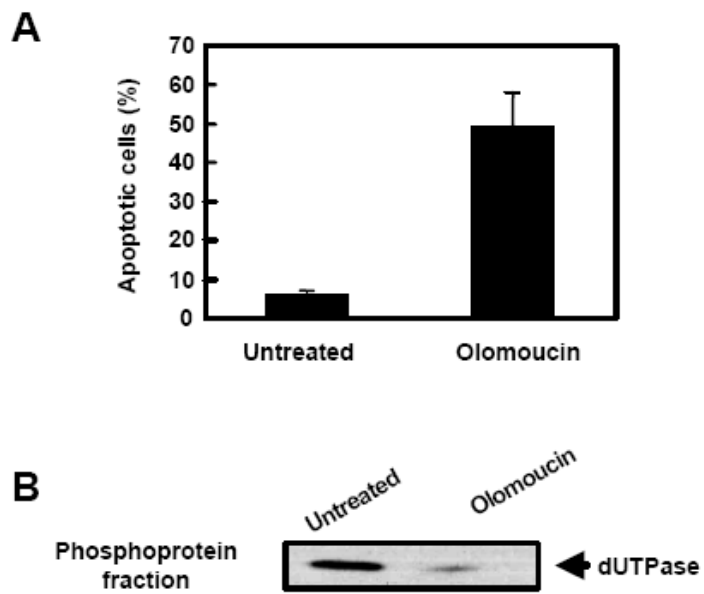


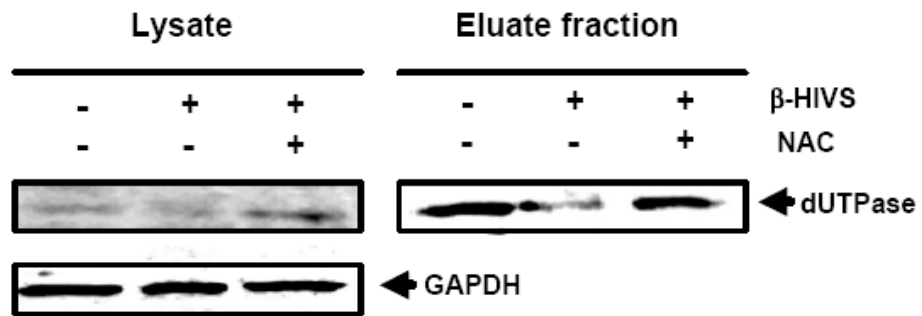
Fig. 4



Figs. 5A and 5B

ACCEPTED

Fig. 6



ACCEPTED MANUSCRIPT

Fig. 7

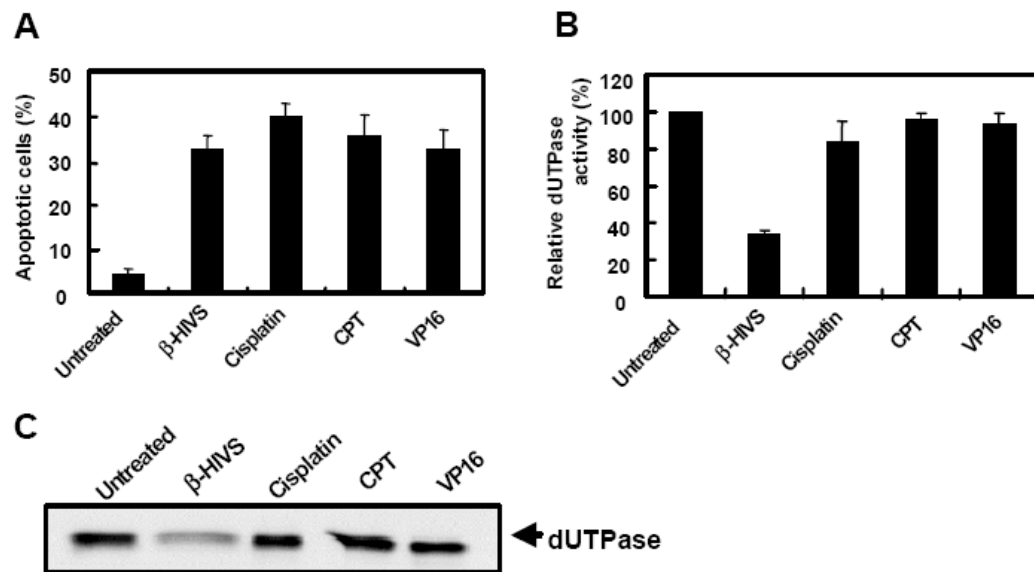
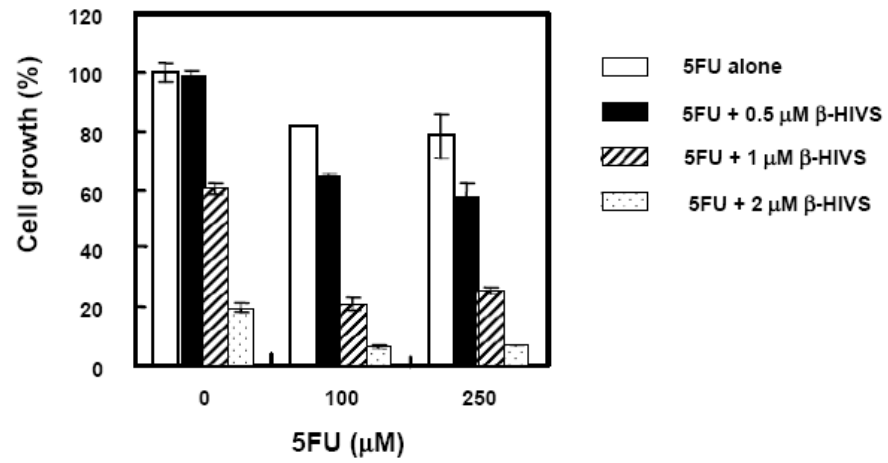


Fig. 8



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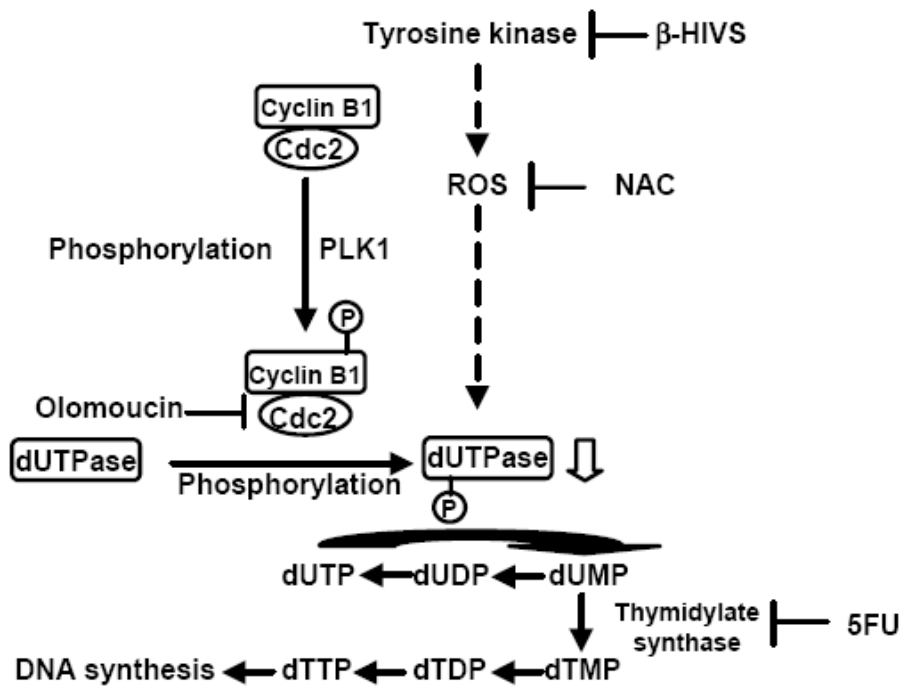


Fig. 9

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