Effect of $\beta$-hydroxyisovalerylshikonin and Cancer Chemotherapeutic agents on Leukemia U937 and Lung Cancer DMS114 Cells \textit{In vitro}

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Abstract: We used U937 (monocyte-like, histiocytic lymphoma) and DMS114 (lung small cell cancer) cell lines to examine the effects of $\beta$-hydroxyisovalerylshikonin ($\beta$-HIVS), a protein tyrosine kinase inhibitor, in combination with cancer chemotherapeutic agents. For U937 cells, combined treatment with $\beta$-HIVS and bufalin had synergistic effects. The combination of $\beta$-HIVS with VP16 showed marginal synergistic to additive effects. Additive to subadditive effects were observed when $\beta$-HIVS was combined with doxorubicin. The above combinations showed few synergistic effects on DMS114 cells.

Key words: chemotherapeutic agents, combination, isobologram, synergism

Introduction

Currently the most effective chemotherapeutic treatment for cancer is combination chemotherapy. However the emergence of resistance can result in the failure of therapy. To improve curative effects and survival rates for cancers, there is an urgent requirement for new anticancer agents and new combination therapies.

$\beta$-HIVS, isolated from the plant, \textit{Lithospermium radix}, inhibits proliferation in human leukemia HL-60 and U937 cells, in melanoma VMRC-MELG cells, in colon adenocarcinoma COLO320DM cells, and in gastric cancer AZ-521 cells, with $IC_{50}$ values that range between $10^{-6}$ and $10^{-8}$ M$^{-1}$. We have found that $\beta$-HIVS specifically inhibited protein tyrosine kinases such as EGFR and v-Src$^{2}$. Recently, Gleevec (STI571), which inhibits the tyrosine kinase activity of BCR-ABL and c-kit, has had remarkable clinical success in treating chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor (GIST)$^{3}$. CML and GIST are caused by mutations in BCR-ABL and c-kit, respectively$^{4,5}$. Furthermore, Gleevec sensitizes BCR-ABL-positive leukemia cells to various antileukemic agents$^{6,7}$.

VP16 is used for various cancers and combined with paclitaxel in lung cancer treatment$^{8}$. Bufalin, one of the main types of bufaienolides in the traditional Chinese medicine chan'su, which is prepared from toad venom, has differentiation induction activity for human leukemia cells HL60$^{9}$ and inhibits growth of HL60$^{10}$. Doxorubicin (adriamycin), one of the first anthracyclines in clinical use, has a broad anti-tumor spectrum, and has been used against hematopoietic malignancies such as lymphoma, myeloma and leukemia, and solid tumors such as breast cancer, ovarian cancer and sarcomas$^{11,12}$.

In the present study, we chose leukemia U937 cells and DMS114 cells, both of which are sensitive to $\beta$-HIVS, to observe the combined effects of $\beta$-HIVS with the anticancer agents.
Materials and Methods

Reagents
Etoposide (VP16), bufalin, doxorubicin (Dox) and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) were purchased from Sigma Chemical Co. (St. Louis, MO). β-HIVS was isolated from Lithospermum radix as described previously.

Cell culture and reagents exposure
U937 (monocyte-like, histiocytic lymphoma) and DMS114 (lung small cell carcinoma) cells were provided by the Japanese Cancer Research Bank (Tokyo, Japan) and maintained in RPMI1640 medium with 10% fetal calf serum in a 5% CO2 humidified incubator at 37°C.

U937 cells were incubated with different combinations of β-HIVS with various chemotherapeutic agents for 72 h after seeding into a 96-well plate; DMS114 cells were allowed to attach to the bottom of a 96-well plate for 24 h and were then treated with various combinations of anticancer agents for another 48 h.

XTT assay
Cell growth was quantitated by the XTT assay. A solution of XTT and phenazine methosulfate (PMS) was added and the culture was continued for a further 4 h. The absorbance was then measured at 492 nm with a Microplate Reader.

Analysis of the cytotoxic effects of β-HIVS combined with chemotherapeutic agents on tumor cells
The cytotoxic effects of β-HIVS with chemotherapeutic agents on the U937 cell line were at the point of IC₅₀ were evaluated by an isobologram. The IC₅₀ was defined as the concentration of agent that produced 50% cell growth inhibition.

The isobologram
The theoretical basis of the isobologram method and the procedure for making isobolograms has previously been described in detail. Based on the dose-response curves of β-HIVS and chemotherapeutic agents, three isoeffect curves were constructed (Fig. 1). If the agents act additively by independent mechanisms, combined data points will lie near the mode I line (hetero-addition). If the agents act additively by similar mechanisms, combined data points will lie near the mode II line (iso-addition). When the data points fell to the left of the envelope, that is, the combined effects were caused by lower doses of the two agents than was predicted, the agent combination was regarded as having a supra-additive effect (synergism). When the points fell to the right of the envelope, that is, the combined effect was caused by higher doses of the two agents than was predicted, but within the square or on the line of the square, the combination was regarded as having a subadditive effect, that is, the combination was superior or equal to a single agent but was less than the additive effect. When the data points were outside the square, the combination was regarded as having a protective effect, that is, the combination was inferior in cytotoxic action to a single agent. Both subadditive and protective effects were regarded as...
Effect of $\beta$-hydroxyisovalerylshikon on Cancer Cells

Fig. 1. An envelope of additivity is constructed from the dose-response curves of two agents (A and B). If experimental data points Pa, Pb, Pc, and Pd fall in the specified area in the diagram, the interactions of two agents can be classified as synergistic, additive, subadditive, and mutually protective, respectively. Mode I line (−), when the dose of Drug A is chosen there remains an increment in effect to be produced by Drug B; Mode IIa line (−), when the dose of Drug A is chosen, an isoeffect curve can be calculated by taking the dose increment of Drug B that gives the required contribution to IC$_{50}$; Mode IIb line (−), when the dose of Drug B is chosen, an isoeffect curve can be calculated by taking the dose increment of Drug A that gives the required contribution to IC$_{50}$. Data points Pa, Pb, Pc, and Pd show synergistic, additive, sub-additive, and protective effects, respectively.

antagonism.

Results

Cytotoxic effect of $\beta$-HIVS on U937 cells

The dose-response curve of $\beta$-HIVS for human leukemia U937 cells is shown in Fig. 2. The IC$_{50}$ of $\beta$-HIVS for U937 cells was estimated to be 0.14 $\mu$M.

Combined effects of $\beta$-HIVS and chemotherapeutic agents on U937 cells

Fig. 3A-C shows isobolograms at IC$_{50}$, based upon the dose-response curves of combinations of $\beta$-HIVS and chemotherapy agents. For simultaneous and continuous exposure of U937 cells to $\beta$-HIVS and bufalin, the results obtained by combination of these agents fell on the left side of envelope (Fig. 3A). This result was interpreted as showing that simultaneous exposure to $\beta$-HIVS and bufalin produced a synergistic effect. In combination of $\beta$-HIVS with VP16, the data points fell in the area of synergism and in the envelope of additivity (Fig. 3 B). In the combination of $\beta$-HIVS with Dox, the data points fell in the area of additivity and subadditivity (Fig. 3 C).
Ying Xu, et al

Fig. 2. The effects of β-HIVS on the growth of U937 cells. Cell growth was quantitated by the XTT assay, as described in "Materials and Methods". The results shown are means±S.D. (bars) of results obtained from three wells in each case. The absence of a bar indicated that S.D. falls within the symbol.

β-HIVS did not synergize with the above chemotherapeutic agents on DMS114 cells

The IC₅₀ value of β-HIVS for DMS114 cells was estimated to be 6.8 µM (results not shown). We selected a β-HIVS concentration of 4 µM, which had virtually no effect on the growth of DMS114 cells, to combine with the chemotherapeutic agents to observe the cytotoxic effects. No synergistic effect was observed by the combination of β-HIVS with bufalin (Fig. 4A). Similarly, no synergistic effects were observed by the combination of β-HIVS with VP16 or with Dox (Fig. 4B and C).

Discussion

β-HIVS, isolated from the Chinese plant Lithospermum radix, was reported to exhibit antitumor activity with IC₅₀ values between 10⁻⁶ and 10⁻⁸ M. It induces apoptosis in leukemia HL60 cells through a mechanism different from that of Fas and etoposide. Recently, we found that β-HIVS inhibits protein tyrosine kinases. In the present study, we used leukemia U937 and solid tumor DMS114 cells, which were sensitive to β-HIVS, and examined the effect of combining β-HIVS with therapeutic agents.

The present study indicated that synergistic and additive effects were observed by the combined treatment of U937 cells with β-HIVS and either bufalin or VP16 and that an additive effect was observed with β-HIVS and Dox combined. By contrast, neither synergistic nor additive effects were observed in DMS114 cells by the same combinations of β-HIVS with bufalin, VP16, or Dox. The difference in the inhibitory effects of β-HIVS combined with anticancer agents between U937 and DMS114 cells might be due to differences in the signaling pathways operating in these cell lines. Both U937 and DMS114 cells express low levels of EGFR. Therefore, the inhibition of cell growth of U937 and DMS114 cells might be, at least partly, due to the inhibition of the protein tyrosine kinase activity of EGFR. In U937 cells, cross-talk between the protein tyrosine kinase pathways
Fig. 3. Isobologram of $\beta$-HIVS in combination with bufalin, VP16, and Dox. 
A, combined with bufalin; B, combined with VP16; C, combined with Dox.
Fig. 4. Effects of \( \beta \)-HIVS in combination with chemotherapeutic agents on the growth of DMS114 cells. DMS114 cells were treated with \( \beta \)-HIVS in combination with the agents indicated for 48 h. Cell growth was quantitated by the XTT assay, as described in "Materials and Methods". The results shown are means \( \pm \) S.D. (bars) of results from three wells in each case. A, combined with bufalin; B, combined with VP16; C, combined with Dox.
and other signaling pathways sensitive to the anticancer agents used in the present study might play an important role in inhibiting cell-growth. Elucidating the signaling pathways operating in DMS114 cells and identifying anticancer agents that inhibit those pathways requires further study.

References


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