Antitumor Effects of Immunotoxins Are Enhanced by Lowering HCK or Treatment with Src Kinase Inhibitors

Xiu-Fen Liu, Laiman Xiang, David J. FitzGerald, and Ira Pastan

Abstract

Recombinant immunotoxins (RIT) are agents being developed for cancer treatment. They are composed of an Fv that binds to a cancer cell, fused to a 38-kDa fragment of Pseudomonas exotoxin A. SS1P is a RIT that targets mesothelin, a protein expressed on mesothelioma as well as pancreatic, ovarian, lung, and other cancers. Because the protein tyrosine kinase family regulates a variety of cellular processes and pathways, we hypothesized that tyrosine kinases might regulate susceptibility to immunotoxin killing. To investigate their role, we used siRNAs to lower the level of expression of the 88 known tyrosine kinases. We identified five tyrosine kinases, INSR, HCK, SRC, PDGFRB, and BMX that enhance the activity of SS1P when their level of expression is lowered by siRNAs. We further investigated the Src family member HCK in this study. Knocking down of SRC slightly increased SS1P killing in A431/H9 cells, but knocking down HCK substantially enhanced killing by SS1P. We investigated the mechanism of enhancement and found that HCK knockdown enhanced SS1P cleavage by furin and lowered levels of Mcl-1 and raised Bax. We then found that Src inhibitors mimic the stimulatory effect of HCK knockdown; both SU6656 and SKI-606 (bosutinib) enhanced immunotoxin killing of mesothelin-expressing cells by SS1P and CD22-expressing cells by HA22 (moxetumomab pasudotox). SU6656 also enhanced the antitumor effects of SS1P and HA22 in mouse xenograft tumor models. Our data suggest that the combination of immunotoxin with tyrosine kinase inhibitors may be an effective way to treat some cancers.

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Introduction

Recombinant immunotoxins (RIT) are chimeric proteins being developed to treat cancer. They are composed of the Fv portion of an antibody linked to a bacterial or plant toxin (1). We produce RITs by linking a 38-kDa portion of Pseudomonas exotoxin A (PE) to Fvs reacting with either CD22 present on the surface of B-cell leukemias and lymphomas or with mesothelin present on mesotheliomas and several other epithelial malignancies (2, 3). HA22, also known as moxetumomab pasudotox, is a RIT that kills CD22-expressing cells. It has been shown to be very active in drug-resistant hairy cell leukemia (HCL); in a phase I trial it had a 90% response rate with 50% of subjects obtaining a complete remission (4). HA22 is also being tested in children with drug-resistant acute lymphoblastic leukemia (ALL) and has produced several complete remissions in that disease, although the response rate is lower than in HCL (5). SS1P is a RIT targeting mesothelin-expressing tumors. When tested by itself, it had low antitumor activity in patients with mesothelioma and ovarian cancer (6, 7), but seems to have more activity when combined with cis-platinium and permeated to treat mesothelioma (8, 9).

Our current efforts are directed at increasing immunotoxin activity in patients by determining how the steps in the pathway by which immunotoxins kill cells are regulated and using this information to identify drugs that will modify these steps and enhance cell killing (10, 11). The mechanism by which RITs kill cells is complex and although much is known it is not fully understood (12–14). Following binding to the receptor on the cell surface, the RIT is internalized by receptor-mediated endocytosis and undergoes processing by furin, which separates the Fv from the toxin. The toxin fragment, which contains a REDL sequence at its C terminus, can then bind to the KDEL receptor and be transported through the Golgi to the endoplasmic reticulum, where it escapes into the cytosol. In the cytosol it catalyzes the ADP-ribosylation and inactivation of elongation factor 2 (EF2) leading to the arrest of protein synthesis. This event initiates the apoptotic cascade by lowering Mcl-1 levels and unleashing Bak to promote apoptosis (11).

Because protein phosphorylation is a major mechanism of protein regulation, and tyrosine kinases are often activated in cancer cells, we have begun to examine the role of protein phosphorylation in the killing of
cells by immunotoxins SS1P and HA22. We have used siRNAs to lower the level of tyrosine kinases and assess the response of cancer cells to SS1P or HA22. We recently reported that lowering expression of the insulin receptor (INSR) enhanced immunotoxin action (15). We chose to examine members of the Src family because Src kinases contribute to important cellular signal pathways, including cell growth, differentiation, cell shape, and migration (16, 17). Many Src family kinases are identified as oncogenes and play important roles in tumor development (18).

We report here that knockdown of HCK, and to a lesser extent, SRC, enhance immunotoxin action by a different mechanism than that regulated by the INSR. We also report that two Src family inhibitors, SU6656 and bosutinib, enhanced immunotoxin killing of cultured cells and that SU6656 synergized with immunotoxins HA22 or SS1P to cause tumor regression in tumor bearing mice.

**Materials and Methods**

**Reagents**

Immunotoxins SS1P and HA22 were purified in our laboratory as described in Pastan and colleagues (19). Anti-β-tubulin, 2-hydroxypropyl-β-cyclodextran, and SU6656 were purchased from Sigma. Bosutinib (SKI-606) was purchased from LC Laboratories. Anti-Bcl2, anti-PARP, anti-cleaved caspase-3, anti-Bax, anti-Bcl-xL, and anti-Mcl-1 were purchased from Cell Signaling Technology. Antiactins were purchased from Abcam. All the siRNAs were purchased from Dharmacon and are listed in Supplementary Table S1. CellTiter-Glo kit was purchased from Promega.

**Cell culture, transfection, and cytotoxicity assay**

Human cell lines A431/H9, A1847, KLM-1, and CA46 were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO2 at 37°C. The cell lines A431/H9 and A1847 were described previously (15). Pancreatic cell line KLM1 was from Dr. Udo Rudloff (NCI, NIH, Bethesda, MD). CA46 was purchased from American Type Culture Collection. Their identities were confirmed by short-tandem repeat analysis within a year.

Cells were transfected at 5,000 cells per well in 96-well plates by the addition of 0.3 μL of 20 μmol/L siRNA and 0.35 μL of DharmaFECT Transfection Reagent 3 in a final volume of 125 μL. After 48 hours, the cells were treated with SS1P for 72 hours. In some experiments, inhibitors were added 1 hour before SS1P. Cell viability was measured with a CellTiter-Glo kit. Cell viability is expressed as the percentage of luminescence with SS1P compared with control without SS1P treatment.

**Western blot analysis**

Cells were washed in PBS and lysed by the addition of lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 10 μmol/L PMSF) on ice for 30 minutes. After high-speed centrifugation, supernatants were analyzed by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to Western blot analysis.

**Internalization and FACS analysis**

Immunotoxin SS1P was conjugated with Alexa Fluor-647 as described previously (15). After SS1P binds to mesothelin on the cell surface, it is internalized into the cells, and the fluorescently labeled cells are detected by fluorescence-activated cell sorting (FACS) analysis. A431/H9 cells were transfected with siRNA for 48 hours in 6-well plates, 1 μg/mL of SS1P-Alexa Fluor-647 was added and incubated at 37°C for indicated time. The control cells are the same cells as the experimental group except there was no addition of SS1P-Alexa Fluor-647. After labeling, the cells were washed with PBS and stripped with glycine buffer containing 0.2 mol/L glycine (pH 2.5) and 1 mg/mL of bovine serum albumin to remove surface-bound SS1P. Cells were then trypsinized and washed with FACS buffer (PBS with 5% FBS and 0.1% NaNO3) and analyzed by flow cytometry using FACScalibur.

**SS1P cleavage**

A431/H9 cells were transfected with siRNA for 48 hours in 6-well plates. One μg/mL of SS1P was added and cells incubated on ice for 30 minutes to saturate SS1P binding. Cells were changed to fresh media and incubated at 37°C for the indicated time before making a total cell lysate and Western blot analysis.

**Real-time PCR**

RNAs were isolated using TRizol reagent (Invitrogen). Reverse transcription and cDNA synthesis were performed with the QuantiTect Reverse Transcription Kit following the manufacturer’s instructions (Qiangen). Human actin primers were used as an internal control. The primer sequences are listed in Supplementary Table S1. The PCR reaction was performed using the QuantFast SYBR Green PCR Master Kit (Qiangen).

**Xenograft tumor model**

1.8 million A431/H9 cells or 10 million CA46 cells with Matrigel (800 μg in 200 μL per mouse) were implanted subcutaneously into a rear leg of athymic nude mice. When tumors reached 100 mm3 (day 6 or 7), 5 or 8 mice in each group were injected intraperitoneally with 300 μg of SU6656 suspended in 20% 2-hydroxypropyl-beta-cyclodextrin. Thirty minutes later, 8 μg SS1P or 6 μg of HA22 was injected intravenously. A total of three doses were injected every other day. Tumor volumes were calculated and measured as previously described (20). The animal protocol was approved by the National Cancer Institute Animal Care and Use Committee. All animal experiments were stopped when tumors reached 1,000 mm3.
Statistical analysis

Statistical analysis of synergy was performed by David Venzon (Biostatistics and Data Management Section, Center for Cancer Research, National Cancer Institute, Bethesda, MD). Repeated measures ANOVA was applied to the changes in successive tumor spherical diameters. Synergy was defined as an interaction effect significantly greater than the sum of the drug and SS1P effect (21).

Results

Tyrosine kinase siRNA library screen

To identify which tyrosine kinases regulate immunotoxin killing, we treated A431/H9 cells, which express high levels of mesothelin, with siRNAs targeting all of the 88 known tyrosine kinases. After 48 hours, we added the immunotoxin (SS1P) and incubated the cells 72 hours to allow apoptosis to occur. We assessed cell death using an assay that measures the cellular level of ATP. Nonspecific siRNA and luciferase siRNA (GL2) served as negative controls (Con), and siMSLN, targeting mesothelin, served as positive control. As expected, siMSLN prevents SS1P killing (Supplementary Fig. S1). We identified 12 siRNAs (BMX, FES, INSR, KDR, KIT, MUSK, PDGFR2, TNKI, HCK, SRC, YESI, and LYN) that enhanced cell killing and three siRNA (MATK, IGF-1R, and EPHAS) that blocked cell killing. Because the siRNA pools consisted of four different oligonucleotides and because the pools could have off-target effects, we then confirmed the results using specific oligos. We found that knockdown of five genes, SRC, HCK, PDGFRb, BMX, and INSR, reproducibly enhanced killing of cells by SS1P. Single oligos targeting FES, KDR, KIT, MUSK, TNKI, YESI, and LYN, respectively, did not reproducibly enhance SS1P toxicity. The results with INSR were described previously (15). Knockdown of Src slightly enhanced SS1P killing in both A431/H9 and KB cells (IC50 value decreased 25% in both cell lines; Supplementary Fig. S2). However, knockdown of HCK gene greatly enhanced SS1P toxicity, with the IC50 decreasing 3-fold as described below.

To demonstrate that the siRNA lowered HCK expression, we analyzed HCK RNA by real-time PCR (RT-PCR) and found HCK RNA was decreased by 70% (Fig. 1A).

![Figure 1](https://example.com/figure1.png)

Figure 1. HCK knockdown specifically enhanced immunotoxin toxicity. A, siRNA siHCK-1 and control siRNA (GL2, luciferase siRNA) were transfected into A431/H9 cells; after 48 hours, RNAs were analyzed by RT-PCR. B, siHCK1, siHCK3, siHCK4, or control siRNAs were transfected into A431/H9 cells for 48 hours. Cell viability was measured by ATP assay after SS1P treated for 72 hours, and the viability (%) expressed as the percentage of luminescence with SS1P compared with control. C, siHCK or control siRNAs were transfected into A1847 cells and the cell viability was measured as in B. D to F, A431/H9 cells were transfected with siCo or siHCK-1; 48 hours later, cells were treated with HB21-PE40 (D), HA22 (E), or cycloheximide (F) for 72 hours, and the cell viability was measured by ATP assay. G to I, CA46 cells were transfected with siHCK-1 or control siRNA (siCo) for 48 hours, RT-PCR (G) or Western blot analysis (H) were analyzed for expression of HCK RNA (G), protein (H), or cells were treated with HA22 and cell viability was determined after 72 hours of treatment by an ATP assay (I).
levels of HCK protein are very low in A431/H9 cells and could not be detected by antibody on Western blot analyses. To assess specificity further, we used siRNAs that target other regions of HCK RNA and found that siHCK-3 and siHCK-4 also enhanced the cytotoxic action of SS1P (Fig. 1B). Because many ovarian cancers express mesothelin, we assessed the ability of siHCK-1 to enhance killing of the ovarian cancer line A1847 by SS1P, and found a marked enhancement in cytotoxicity in that cell line (Fig. 1C).

To determine whether the HCK knockdown effect would enhance killing of A431/H9 cells by an immunotoxin targeting another receptor on A431/H9 cells, we treated the cells with immunotoxin HB21-PE40 (anti-TFR-PE40) that targets the human transferrin receptor and found that its cytotoxic activity was greatly increased upon knockdown of HCK (Fig. 1D). We also tested the effects of HCK knockdown using immunotoxin HA22, which targets CD22, not expressed on A431/H9 cells, and found that cytotoxicity was not induced by HCK knockdown (Fig. 1E). We also studied the effects of HCK knockdown using cycloheximide, which inhibits total protein synthesis. As shown in Fig. 1F, knockdown of HCK did not stimulate cycloheximide-induced cell killing. These results demonstrate that knockdown of HCK enhances the activity of immunotoxins that have specific receptors on target cells and does not provoke nonspecific internalization or cell killing of a nontargeted immunotoxin.

To determine whether siHCK knockdown would enhance killing of a lymphoma cell line, we used CA46 cells, which express CD22 and the immunotoxin HA22 that targets CD22-expressing cells. We transfected CA46 cells with siHCK-1 and found it decreased HCK RNA and protein levels by about 50% (Fig. 1G and H). Because transfection efficiencies are often low using non-adherent cells, we ascribe this moderate degree of level of

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Figure 2. HCK knockdown did not affect SS1P internalization, but affected SS1P cleavage. A, SS1P internalization after knockdown of HCK. A431/H9 cells were transfected with siHCK-1 for 48 hours and SS1P-Alexa Fluor-647 was added and incubated at 37°C for indicated time before FACS analysis. B, SS1P fluorescence intensity (geometric mean) analyzed by FACS (shown in A) plotted over time. C, SS1P cleavage after knockdown of HCK. A431/H9 cells were transfected with siHCK-1 for 48 hours, 1 μg/mL of SS1P was added and incubated on ice for 30 minutes, then chased at 37°C for indicated time. Total cell lysates were analyzed with polyclonal antibody anti-Pseudomonas exotoxin A by Western blot analysis. PE35 is the cleaved form of SS1P. D and E, representative Western blot analysis was scanned and analyzed by NIH ImageJ, the relative intensity of heavy chain-toxin SS1P (D) or PE35 (E) was plotted. F, protein synthesis inhibition assay: A431/H9 cells were transfected with siHCK-1 and control GL2 for 48 hours, SS1P was added for 20 hours; protein synthesis was measured by 3H-leucine incorporation.
knockdown to poor transfection efficiency. Nevertheless, it was sufficient to allow cytotoxicity studies and as shown in Fig. 1I, the cytotoxic activity of HA22 was significantly stimulated by HCK knockdown; the IC$_{50}$ value shifted from above 5 to 2.4 ng/mL. We conclude that HCK knockdown can enhance immunotoxin action on several different cell types.

**HCK knockdown stimulates SS1P processing**

To investigate where in the intoxication pathway the stimulatory effect of HCK knockdown was occurring, we examined several different steps in the intoxication pathway. We began by examining the uptake of SS1P using SS1P labeled with a fluorescent dye as previously described (15). We found that the uptake of SS1P was not increased and was actually slightly decreased upon HCK knockdown (Fig. 2A and B). These data show that enhanced entry of SS1P does not explain the increase in cytotoxic activity.

The next step in the pathway is the processing of SS1P by furin, which cleaves the toxin between residues 279 and 280 of *Pseudomonas* exotoxin A and generates an Fv fragment and a 35-kDa fragment of *Pseudomonas* exotoxin A containing the ADP-ribosylating activity (14). Although furin protein levels did not increase after knockdown of HCK; the amount of the 35-kDa fragment increased about 2- to 3-fold at the 5-, 20-, and 60-minute time points when HCK is knocked down (Fig. 2C–E). An increased amount of processed immunotoxin should lead to more of the toxin fragment delivered to the endoplasmic reticulum and the cytosol, a more rapid inactivation of EF2 and a decrease in protein synthesis. Protein synthesis in SS1P-treated cells was measured by $^3$H-leucine incorporation (11). Figure 2F shows that in cells treated with increasing amounts of SS1P, there is a greater decrease in leucine incorporation in cells treated with siRNAs for HCK compared with a control siRNA. IC$_{50}$ decreased from 2.5 to 1.2 ng/mL. This indicates that the fragment of *Pseudomonas* exotoxin A produced by increased processing can reach the cytosol and inhibit protein synthesis to a greater extent.

**Knockdown of HCK decreases Mcl-1 levels and increases Bax levels**

To investigate whether knockdown of HCK also affected the levels of proteins involved in apoptosis and previously shown to control immunotoxin killing (11), we examined the levels of Bak, Bax, Mcl-1, Bcl2, and BclXL in A431/H9 cells by Western blot analysis. Figure 3A shows that the level of the antiapoptotic protein Mcl-1 decreased about 5-fold and the level of the proapoptotic protein Bax was increased about 4-fold after knockdown HCK. The changes in BclXL, Bcl2, and Bak were much smaller. We examined the markers of apoptosis PARP and cleaved caspase-3 (Fig. 3B) and found that HCK knockdown by itself substantially decreased full-length PARP levels. When knockdown was combined with SS1P treatment, PARP levels were further decreased and cleaved caspase-3 levels increased, as would be expected in cells undergoing apoptosis.

**Src family kinase inhibitors**

To investigate whether we could enhance SS1P or HA22 activity with a tyrosine kinase inhibitor, we tested SU6656 and SKI-606 (bosutinib), known to inhibit members of the Src family (22, 23). We found that combining the Src kinase inhibitor (SU6656) with SS1P gave a synergistic inhibition of cell growth compared with the addition of either agent alone, on three different epithelial cancer lines A431/H9, A1847, and KLM-1, a pancreatic cancer line (Fig. 4A–C). SU6656 also enhanced the cytotoxic activity of HA22 on the CA46 lymphoma cell line (Fig. 4D). Bosutinib/SKI606 synergistically enhanced killing of A431/H9 cells by SS1P and CA46 cells by HA22 (Fig. 4E and F, respectively).

**SU6656 enhanced immunotoxin activity in mice xenografts**

To determine whether SU6656 could enhance the antitumor activity of SS1P, mice were implanted with A431/H9 cells and treatment was initiated on day 6. Mice received either SS1P or SU6656 or SS1P and Su6656 in combination. As shown in Fig. 5A, when mice were treated with SU6656, there was a minimal change in tumor growth compared with control. SS1P treatment significantly reduced tumor growth, and tumor growth was retarded further when the two agents were combined. Statistical analysis indicated that the drugs showed a synergistic effect on days 12, 14, and 16 ($P < 0.01$).

We tested the effect of SU6656 in mice bearing CA46 lymphomas and found that SU6656 alone had very little effect on the growth of the tumors but greatly enhanced the antitumor activity of HA22 (Fig. 5B). The asterisks indicate that on days 14, 16, and 19, the combination had a
synergistic effect on inhibiting tumor growth \((P = 0.011, P < 0.0001, \text{and } P = 0.001, \text{respectively})\).

**Discussion**

We have used siRNAs targeting members of the 88 tyrosine kinases to identify genes that can regulate immunotoxin killing, and found that knockdown of several tyrosine kinase genes enhanced killing of A431/H9 cells. These include HCK, which produces a large enhancement and SRC whose effect is less. There are nine members of the Src family (Src, Yes, Lyn, Fyn, Blk, Fgr, Lck, Hck, and Frk); the other seven members were not confirmed to be active in our study. Because the substrate specificities of the various family members overlap, we were surprised that we did not find similar results with knockdown of other family members. It is possible that Hck is specifically associated with proteins that regulate toxin action or that Hck has a substrate specificity that explains its selective effect. It is also possible that in cells other than A431/H9, other members of the Src family can regulate immunotoxin action. This can be investigated by performing knockdown experiments with the other Src family members in different types of cells. However, the finding that HCK knockdown also enhances killing of A1847 ovarian cancer cells and CA46 lymphoma cells indicates that Hck has an important regulatory role in several types of cancer.

We have previously shown that knockdown of the INSR can enhance immunotoxin action by increasing the processing of the immunotoxin by furin (15). Knockdown of HCK similarly increased the furin cleavage and increased the amount of 35-kDa Pseudomonas exotoxin A fragment, indicating that Hck also modulates immunotoxin processing. Hck has additional effects not seen with INSR knockdown. It has major effects on the levels of two important proteins that regulate apoptosis: lowering HCK decreases the antiapoptotic Mcl-1 protein and elevates the proapoptotic Bax protein. This is the first report that HCK can regulate pro/antiapoptotic protein levels. There are studies on other Src family kinases; for example, Src and Fyn play a role in the antiapoptotic response in fibroblasts (24) and knockdown of Lyn induced caspase-8 activation.

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**Figure 4.** SU6656 and SKI-606 stimulated SS1P and HA22 toxicity in cell lines. Of note, 5,000 cells (A431/H9, A1847 and KLM-1) or 10,000 cells of CA46 were plated in 96-well plates overnight. SU6656 (A–D) or SKI-606 (E–F) at indicated concentrations was added to cultures 1 hour before SS1P or HA22 addition. After 72 hours, cell viability was measured by an ATP assay. Asterisks (*) indicate synergistic effects of immunotoxin and SU6656 or SKI-606. SS, SS1P; Su, SU6656; and SKI, SKI606.
in a mesothelioma cell line (25). The mechanism by which HCK regulates the levels of apoptotic proteins will be studied in the future.

The protein encoded by the HCK gene was originally identified in hematopoietic cells (26), and its function is being studied in lymphoid and myeloid cells (27, 28). It is now known to be expressed in many different tissues and cell types (http://www.genecards.org/cgi-binocarddisp.pl?gene=HCK), although its role in nonhematopoietic cells has not been extensively studied. Our studies have identified an important role for Hck in regulating the levels of Mcl-1 and Bax in an epithelial cancer cell line and the mechanism by which this occurs needs further study.

The major goal of our experiments was to identify therapeutic agents that could be used in patients to enhance immunotoxin action. Recently, Src family kinases were found to be hyperactivated in malignant mesothelioma specimens and cell lines compared with normal mesothelial cells (29), and Src activation in mesothelioma samples was found to correlate with a more advanced pathologic stage and the presence of metastasis (30). Also, Lck expression was found to be correlated with resistance to dexamethasone in chronic lymphocytic leukemia (31). Moreover, Src kinase activity was found to be constitutively high in many human B-lymphoma cell lines and primary lymphoma samples. The inhibitors of SFKs, PP1, and PP2 inhibited the proliferation of human B lymphomas in a dose-dependent manner (32). We identified two Src kinase inhibitors bosutinib (SKI-606) and SU6656 that show promise. We found that both of these inhibitors would replicate the effect of HCK knockdown and enhance immunotoxin killing of both epithelial and lymphoma cells. We performed antitumor experiments in mice with SU6656 and found that using a dose of SU6656, which by itself had no antitumor activity, produced synergistic antitumor effects when combined with an immunotoxin targeting mesothelin on an epithelial cancer or an immunotoxin that targets CD22 on B-cell malignancies (Fig. 5). Both of these agents are in clinical trials and HA22 as a single agent has produced complete regression in several children with ALL (5). The combination of HA22 and bosutinib, which is approved for chronic myelocytic leukemia (http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm318203.htm), might be useful for treating children with ALL who have a poor response to HA22.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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