Enhancement of glioblastoma cell killing by combination treatment with temozolomide and tamoxifen or hypericin


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Object. The chemotherapeutic agent temozolomide has demonstrated antitumor activity in patients with recurrent malignant glioma. Because responses are not enduring and recurrence is nearly universal, further improvements are urgently needed.

Methods. In an effort to increase the clinical activity of temozolomide, the authors investigated whether its antitumor activity could be enhanced by adding tamoxifen or hypericin, two drugs that are known to inhibit the activity of protein kinase C. Human glioblastoma multiforme cell lines A172 and LA567 were treated with combinations of temozolomide and tamoxifen or hypericin in vitro, and cell survival was analyzed using various methods. Tamoxifen and hypericin were able to greatly increase the growth-inhibitory and apoptosis-stimulatory potency of temozolomide via the downregulation of critical cell cycle–regulatory and prosurvival components. Furthermore, with the use of an in vivo xenograft mouse model, the authors demonstrated that hypericin was able to enhance the antiglioma effects of temozolomide in the in vivo setting as well.

Conclusions. Taken together, analysis of the results indicated that combination therapy involving temozolomide and tamoxifen or hypericin potently inhibited tumor growth by inducing apoptosis and provided an effective means of treating malignant glioma.

Key Words • glioblastoma multiforme • temozolomide • tamoxifen • hypericin • Bcl-2 • mouse

Despite aggressive treatment, malignant GBM has remained difficult to treat, and its overall response to treatment has remained poor, as has outcome in patients harboring this lesion.30,34,41,44 Clearly, new treatment strategies are urgently needed. One of the more potent antiglioma chemotherapeutic agents with demonstrated efficacy is temozolomide (Temodar [in the US], Temodal [globally]; Schering Corporation, Kenilworth, NJ), an orally administered alkylating agent that is well absorbed and readily crosses the blood–brain barrier.31,47 Meaningful anti–GBM activity of temozolomide has been demonstrated in clinical trials when used alone or in combination with other drugs such as adenovirally delivered tumor necrosis factor–α,46 thalidomide,2 etoposide,27 irinotecan,1,19,25,36 or radiotherapy.8 However, although temozolomide is effective in delaying disease progression and maintaining health-related quality of life, durable responses have not yet been documented and the patients eventually die of this disease.15,46 To explore additional ways of increasing temozolomide-induced tumor-cell killing, we investigated whether its anti–GBM efficacy could be enhanced by two drugs that are known to inhibit the important cell survival enzyme, PKC.

It is known that highly malignant tumor cells can escape cell death by overexpressing various prosurvival factors, such as antiapoptotic proteins, repair enzymes, or various protein kinases that are involved in intracellular signal transduction—such as PKC or mitogen-activated protein kinases.22,24,35 For example, increased activity of members of the PKC family has been implicated in cancer
and is frequently found in malignant GBM cells compared with untransformed glial cells. High PKC activity correlates strongly with increased tumor cell proliferation and migration, and provides a survival advantage because of PKC’s ability to stimulate the synthesis of DNA repair enzymes and the antiapoptotic protein Bcl-2. In this regard, the authors of previous works have found that the growth rates of malignant GBM cells are exquisitely sensitive to modulation by PKC in vitro and that the use of PKC inhibitors to downregulate or directly inhibit PKC activity leads to reduced GBM cell proliferation and apoptosis. Additionally, PKC inhibitors have been found to enhance the cytotoxic effect of the topoisomerase inhibitor, Irinotecan, in GBM cells. Therefore, PKC inhibitors may act as chemosensitizers for GBM and potentiate the activity of other chemotherapeutic drugs.

In our work, we have used two different drugs, tamoxifen and hypericin, which are known to exert pleiotropic molecular effects, most notably the potent inhibition of PKC. Although tamoxifen is primarily used as an estrogen antagonist in the treatment of breast cancer, the authors of other studies have reported finding additional estrogen receptor–independent functions that involve the inhibition of various cellular components, including primarily PKC. When high-dose tamoxifen administration was investigated for use in recurrent high-grade gliomas, a 20 to 40% response rate was reported. In a small recently completed Phase II study of concurrent continuous tamoxifen and temozolomide for recurrent malignant astrocytic gliomas, no improvement was reported, although the authors noted that their study was perhaps too small to detect differences and that the selected doses and schedules were not optimized.

Hypericin is a naturally occurring photosensitizer found in Hypericum perforatum plants, commonly known as St. John’s wort. The investigators of several studies have established its potent in vitro and in vivo antineoplastic activity, at least some of which apparently occurs in the absence of light activation. At the molecular level, it was found that hypericin-induced tumor cell death appears to involve several intracellular signaling pathways, including the inhibition of PKC and mitogen-activated protein kinases p42/p44. Several groups investigating GBM cell lines have demonstrated that this compound is able to exert potent antiglioma effects and to inhibit motility and invasion of GBM cells in vitro.

In consideration of the inhibitory effects of tamoxifen and hypericin on intracellular growth and survival pathways, we sought to determine whether these compounds might enhance the antitumor effects of temozolomide and could, thus, be considered when designing more effective regimens of anti-GBM therapy. In the present study we demonstrate that both of these drugs were able to greatly increase the antiproliferative and apoptosis-inducing efficacy of temozolomide on GBM cells in vitro. In addition, hypericin was able to significantly (p < 0.05) increase temozolomide’s antitumor effect in a xenograft mouse model in vivo.

Materials and Methods

Drug Preparation

Temozolomide was obtained from Schering Plough (Kenilworth, NJ), tamoxifen from Sigma Chemical Co. (St. Louis, MO), and hypericin from Dr. Leonard Liebes (New York University, New York, NY). Temozolomide was dissolved in PBS to generate a stock solution of 25 mM; tamoxifen was dissolved in ethanol to generate a stock solution of 10 mM; and hypericin was dissolved in dimethyl sulfoxide to generate a stock solution of 10 mM.

Cell Cultures

The human GBM cell lines A172 and U87 were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco modified Eagle medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (GIBCO) in a humidified incubator at 37°C in a 5% CO2 atmosphere. Additionally, LA567, a multiply passaged primary cell culture, was established from tissue obtained at surgery in a patient with previously untreated GBM. This tumor tissue was cut into 1-mm3 sections, incubated with 1% trypsin, and transferred to culture medium.

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Methylthioindazole Assay

The effects of various drug treatments on GBM tumor cell growth were evaluated by the MTT assay as previously described. Briefly, GBM cells were seeded at a density of 2000 cells per well in 96-well plates and were allowed to adhere overnight. Various concentrations of the different drugs were added individually or as combinations in replicates of three. The MTT assays were performed after 48 hours of drug treatment.

The TUNEL Assay

The TUNEL technique was used to detect 3’-OH ends of the DNA exposed during the interchromosomal cleavage process of apoptosis. The assay was performed using the ApopTag apoptosis detection kit (Intergen, New York, NY) according to the manufacturer’s protocol.

Detection of DNA Laddering

The presence of interchromosomal DNA cleavage (DNA laddering) was determined as described previously. The concentration of DNA in each sample was determined, and equal amounts of DNA were subjected to electrophoresis in a 1.2% agarose gel containing 0.5 mg/ml ethidium bromide. The DNA laddering was visualized by ultraviolet fluorescence.

Western Blot Analysis

The cells were lysed in radioimmunoprecipitation buffer, and equal amounts of protein from each sample were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis as described. Monoclonal antibodies against Bax and Bcl-2 were purchased from Santa Cruz Biotech (Santa Cruz, CA) and used according to manufacturer’s recommendations. As secondary antibodies, we applied horseradish peroxidase–conjugated goat anti–mouse antibodies (Vector Laboratories, Burlingame, CA). Image development was performed with the addition of chemiluminescent detection solution (Amersham Biosciences Corp., Piscataway, NJ) and exposure to Hyperfilm (Amersham).

In Vitro Kinase Assay for CDK

In vitro kinase reactions of CDK were performed, as described in detail elsewhere. Briefly, for the immunoprecipitation of CDK complexes, 250 µg of cellular lysate was incubated with 1.2 µg of anti–cdk2 antibody. The kinase reactions were incubated on a rocking platform at room temperature for 30 minutes. The reaction products were separated on a 12% acrylamide gel and exposed to CL-XPosure film (Pierce, Rockford, IL). After exposure, the gel was stained with Coomassie brilliant blue R-250 to verify equal loading in each lane. The kinase reactions were repeated several times with independent lysates.
We investigated the width U87 GBM cells. Approximately 3 to 4 weeks later, a palpable tumor had formed, and drug treatment was initiated. The mice treated with temozolomide alone received 5 mg/m²/day dose for 5 days as a single cycle. Those treated with hypericin alone received 0.2 mg/kg/day on alternate days until the experiment was terminated. Those treated with combination temozolomide and hypericin received 5 mg/m²/day of temozolomide as a single cycle, whereas hypericin was delivered at 0.2 mg/m²/day dose every alternate day until the experiment was terminated. All drugs were administered orally via a rounded-tip animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY) placed directly into the stomach. The size of the tumor in each animal was measured every 2 to 3 days with calipers, and tumor volume was calculated according to the formula V = length × width × height × 0.5, where V indicates tumor volume.

**Immunohistochemical Analysis of Tumor Tissue**

When the mice were killed, tumor tissue was collected, embedded in tissue-Tek, snap frozen, and stored at −80°C. Sections of 7-μM thickness were cut using a cryostat, dried, and fixed in acetone, quenched in 0.3% H2O2, and then blocked in 5% nonfat milk for 30 minutes at room temperature. The sections were incubated overnight in primary antibody (mouse monoclonal anti–Bcl-2) at 4°C. As the secondary antibody, we used a biotinylated horse anti–mouse immunoglobulin (Vector Laboratories) for 1 hour at room temperature, which was followed by Vectastain ABC (Vector Laboratories) according to the manufacturer’s instructions. Hematoxylin was used as the counterstain. Negative controls were set up with the omission of the primary antibody.

**Results**

To determine whether the antiproliferative effects of temozolomide on GBM cells could be enhanced by hypericin or tamoxifen, we incubated cells with various combinations of these drugs for 48 hours and performed standard MTT assays to determine the viability of these cells after treatment. Human GBM cell lines A172 and LA567 were used for these experiments. As shown in Fig. 1, treatment of A172 cells with temozolomide (1 mM) and tamoxifen (10 μM) or hypericin (10 μM) alone resulted in a small but significant reduction of cell survival. Furthermore, the combination of temozolomide with tamoxifen or hypericin reduced cell survival even further. Similarly, in LA567 cells the combination effects of combined drug treatment were significantly (p < 0.05) stronger than treatment with either drug alone (Fig. 1).

Consistent with the results of the MTT assays, morphological examination showed that combination drug treatments appeared to be more cytotoxic than the individual drug treatments (Fig. 2). Typically A172 cells present as large, flat cells with broad processes. In the presence of temozolomide or hypericin alone, the cells were fewer in number and rounded in appearance, whereas the combination of these drugs led to the appearance of rounded, floating cells with prominent dark nuclei. Similar results were observed in the testing of the LA567 cell type when temozolomide and tamoxifen were combined (Fig. 2).

We next sought to determine the molecular basis for the observed drug combination effects and therefore investigated two critical parameters of cell growth and survival—that is, cell cycle and apoptosis. To establish whether drug treatments would impinge on cell cycle–regulatory processes, we determined the activity of one of the CDKs, which are the essential regulators of cell cycle progression, also called the cell-cycle engine. We investigated a representative member of these CDKs, cdk1–cyclin B complex, the master regulator of G2/M progression. As demonstrated in Fig. 3, treatment of A172 cells with the individual drugs alone had very little or no effect on CDK activity. In contrast, however, temozolomide combined with tamoxifen or with hypericin greatly reduced the enzymatic activity of this critical cell-cycle regulator, consistent with the stronger combination drug effects that we found in MTT assays.

To establish whether the antiproliferative activities of these drug combinations were mediated primarily through cytostatic effects or were followed by cytotoxic consequences, we determined whether the observed cell cycle–inhibitory drug effects led to apoptosis of these tumor cells. We employed two common methods to determine apoptosis, the TUNEL and DNA laddering assays. The results from the TUNEL assay (Fig. 4) clearly indicated that combined drug treatment (temozolomide with tamoxifen or hypericin) led to a higher rate of apoptosis in both A172 and LA567 cells than either individual drug treatment alone. Similarly, analysis of DNA laddering (Fig. 5) confirmed that combined drug treatment caused substantially greater apoptotic cell death than individual drug treatment. Taken together, these results clearly demonstrated increased cytotoxicity when tamoxifen or hypericin is combined with temozolomide in vitro.

In general, apoptosis is regulated by a fine-tuned balance of antiapoptotic and proapoptotic proteins. In this regard, the ratio of expression of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 is known to exert decisive influence on the initiation of apoptosis. We therefore investigated the Bcl-2–Bax ratio in our drug-treated cells by performing Western blot analysis with specific antibodies. As shown in Fig. 6, although the overall amount of Bax was maintained in drug-treated cells, the expression levels of Bcl-2 were substantially reduced in cells treated with both drug combinations. Therefore, the ratio of Bcl-2 to Bax was greatly shifted in favor of the proapoptotic protein Bax, providing a basis for the observed increase of apoptosis in these drug treatment conditions.

Because the aforementioned drug effects occurred under in vitro conditions, it was important to determine if such effects could also be observed under in vivo conditions. Toward this goal, we used a xenograft mouse tumor model with subcutaneously implanted human U87 GBM cells. These animals were treated with either temozolomide or hypericin alone or with a combination of temozolomide and hypericin, and tumor growth was compared with that observed in tumor-bearing animals that did not receive drug treatment. The results, shown in Fig. 7, reveal that either drug alone exerted tumor-inhibitory effects; however, the combined application of both drugs exerted stronger antitumor effects than either drug by itself. Thus, similar to what we observed in vitro, the combined drug treatment was more efficacious in vivo as well.
We next investigated whether combined drug treatment in vivo would cause apoptotic cell death similar to that found in vitro. Tumor sections obtained in untreated control and drug-treated animals were analyzed for any changes in Bcl-2 and Bax protein levels and for the presence of apoptotic cells. As shown in Fig. 8A, tumors in untreated animals displayed extensive staining for Bcl-2, indicating that the implanted GBM cells continued to express high levels of this antiapoptotic protein in vivo. In comparison, tumors in animals treated with temozolomide or hypericin alone had noticeably less intense staining. Remarkably, very few positively stained cells could be found in resected tumors in animals that received combination treatment of temozolomide and hypericin (Fig. 8A). Immunohistochemistry for Bax protein was positive for both the treated and untreated control group (Fig. 8B).

These results were consistent with those we obtained by Western blot analysis, which revealed the strong down-regulation of Bcl-2 protein and no change in Bax protein after the cells were treated with this drug combination in vitro.

Similarly, when the extent of apoptotic cell death was determined by TUNEL staining of tumor sections, we found that this process correlated with the in vitro results as well. As shown in Fig. 9, tumors obtained in control animals that had not received drug treatment exhibited only a few apoptotic cells, whereas tumors removed from animals treated with a single drug displayed a somewhat higher level of apoptotic cell death. In comparison, however, the tumors from animals that received the combination drug treatment of temozolomide and hypericin clearly exhibited a considerably greater extent of apoptosis.

Fig. 1. Bar graphs showing enhancement of cell growth inhibition when temozolomide (TMZ) is combined with tamoxifen (TAM) or hypericin (HY). The A172 and LA567 GBM cells were exposed to temozolomide (1 mM) and tamoxifen (10 μM) or hypericin (10 μM). After 48 hours, MTT assays were performed, and cell growth and survival were determined by optical density (OD) reading at 560 nm, as described in Materials and Methods.

Fig. 2. Photomicrographs demonstrating morphological changes in GBM cells upon treatment with temozolomide in combination with tamoxifen or hypericin. The A172 and LA567 GBM cells were exposed to temozolomide (1 mM), tamoxifen (10 μM), and hypericin (10 μM) individually or in combination as indicated. After 48 hours, photographs were taken under the microscope. Representative sections are shown. AEC peroxidase, original magnification × 20.
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**Fig. 3.** Decreased CDK activity in cells treated with temozolomide in combination with tamoxifen or hypericin. The A172 and LA567 GBM cells were exposed to temozolomide (1 mM), tamoxifen (10 μM), and hypericin (10 μM) individually or in combination as indicated. After 48 hours, cells were lysed, and the activity of the key G2/M regulator, the cyclin B–cdk1 complex, was determined by in vitro kinase reactions. These reactions were separated by polyacrylamide gel electrophoresis and exposed to x-ray film. The top panel shows an autoradiograph reflecting the amount of radioactivity that was incorporated into histone H1, which was used as the in vitro substrate for the immunopurified CDK complex (indicated by the arrow marked 32P-H1). Below is the same gel stained with Coomassie blue to show that the same amount of substrate histone H1 protein was used in each reaction (indicated by the arrow marked H1). Co = control.

**Fig. 4.** Photomicrographs showing increased apoptosis in cells treated with temozolomide combined with tamoxifen or hypericin revealed by TUNEL assay. The A172 and LA567 GBM cells were exposed to temozolomide (1 mM), tamoxifen (10 μM), and hypericin (10 μM) individually or in combination as indicated. After 48 hours, the cells were stained using the TUNEL technique to show apoptotic cells. Note that the red stain is indicative of apoptosis and is strongest in cells treated with temozolomide and tamoxifen and temozolomide and hypericin. AEC peroxidase, original magnification × 20.
Thus, taken together, our results demonstrated that the combination of temozolomide with hypericin potently inhibited GBM cell growth in vitro and in vivo and that these effects closely correlated with the potent downregulation of Bcl-2 and the effective apoptosis-related induction of cell death.

Discussion

Although the alkylating agent temozolomide has greatly extended our armamentarium in the treatment of malignant glioma, therapeutic responses are not enduring and the vast majority of patients die of this disease. In an effort to increase the clinical efficacy of temozolomide, we studied whether the antitumor effects of this drug could be enhanced by concurrent application of other drugs that have demonstrated antiglioma activity, tamoxifen and hypericin. Both of these drugs exert pleiotropic effects on intracellular growth and survival pathways, in particular the inhibition of PKC. Because PKC activity is frequently elevated in malignant glioma and correlates with the pro-

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We hypothesized that inhibition of this growth-stimulatory kinase by tamoxifen or by hypericin might enhance the antitumor efficacy of temozolomide.

Our analyses demonstrated that combination treatment of GBM cells in vitro, as well as of xenograft GBM in experimental animals in vivo, exerts substantially greater antiproliferative and apoptosis-stimulatory effects than treatment with the individual drugs alone. Although our in vitro concentrations of tamoxifen and hypericin were much higher than the concentrations used in clinical trials, the increased concentrations could simply be the result of the use of the MTT assay to measure in vitro cytotoxicity. We have subsequently found that temozolomide is much more effective in a colony-forming assay than in an MTT assay (unpublished data).

One of the underlying molecular events affecting this process appears to be the potent downregulation of Bcl-2, a critical antiapoptotic and chemoprotective protein, which is observed in tumor cells treated with our combination drug regimen in vitro as well as in vivo. It has been well established that the ratio of the intracellular amount of the two proteins, Bcl-2 (an antiapoptotic protein) and Bax (a proapoptotic protein), is critical for subsequent cell survival and cell death. On the one hand, tilting the intracellular balance toward an increased Bax–Bcl-2 ratio, which can occur through elevated synthesis of Bax or through decreased synthesis of Bcl-2, will favor apoptosis and thus reduce the rate of survival. On the other hand, increased levels of Bcl-2 protein, which are observed in many tumor cells, decidedly contribute to increased tumor cell resistance to various chemotherapeutic interventions.

In our experiments, we observed a dramatic decrease of Bcl-2 levels in cells that were treated with temozolomide and tamoxifen or temozolomide and hypericin. Thus, it is reasonable to assume that the consequent greatly increased ratio of Bax protein caused enhanced cell death. Indeed, this appears to be the case, as we observed substantially increased apoptotic cell death in these conditions.

Importantly, we found that the in vitro drug effects also occur in an animal model in vivo. Because temozolomide and hypericin exerted greater in vitro effects on cytotoxicity, inhibition of Bcl-2, and evidence of apoptosis than temozolomide and tamoxifen, we used this combination for our in vivo experiments. We found that tumors from animals that received temozolomide combined with hypericin also displayed dramatically reduced expression of Bcl-2 and, at the same time, exhibited a significantly increased rate of apoptosis. Such concordance of in vivo and in vitro results encourages the hope that such effects might also occur in the clinical setting in patients with GBM; this therefore supports the notion that these drug combinations should be further evaluated in clinical trials. Hypericin has been used as single-agent chemotherapy in a Phase I/II recurrent malignant glioma clinical trial and was shown to cause radiographically verified tumor regression in selected patients. Hypericin was given orally and was found to be well tolerated (L. F. Leibes, personal communication). Therefore, it should be possible to ex-

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**Fig. 7.** Graph showing the drug’s effects on xenograft tumor growth in mice. The nude mice underwent subcutaneous implantation of U87 GBM cells. After palpable tumors had developed, the animals were separated into four treatment groups: 1) control group (PBS treatment only); 2) treatment with temozolomide only (5 mg/m^2/day for 5 days total); 3) treatment with hypericin only (0.2 mg/kg/day, every other day); 4) treatment with a combination of temozolomide + hypericin (same dose and schedule as above). Shown is the average increase in tumor volume over time (eight samples; values are ± the standard deviations). The difference in tumor size between the control (PBS) group and the temozolomide-treated group, as well as that between the control and the hypericin-treated group, was statistically significant (p < 0.05) at the end of the experiment. Similarly, the difference in tumor size between the animal group that received combination drug treatment (temozolomide and hypericin) was statistically significantly different (p < 0.05) and those animals that received individual drug treatment of temozolomide or hypericin alone.
tend its use in combination therapy with other chemotherapeutic agents.

Considering the apparently critical involvement of Bcl-2 in the aforedescribed processes, it might also be worthwhile to investigate treatment approaches in which temozolomide is combined with suppressive manipulations of intratumoral Bcl-2 protein levels. It has been demonstrated, for example, that various methods to downregulate Bcl-2 in tumor cells, such as antisense approaches or RNA interference, can be applied successfully to block Bcl-2 expression and sensitize tumor cells to apoptotic cell death.\textsuperscript{3,25,42} Therefore, the combination of such methods with temozolomide treatment could prove particularly beneficial in GBM, which is known not only to harbor elevated levels of this antiapoptotic and chemoprotective protein, but also appears to increase further its production during the shift from initial to recurrent malignancy.\textsuperscript{19,39}

In any case, our results demonstrated that the combination of temozolomide with hypericin efficiently suppressed Bcl-2 expression in GBM cells. The precise molecular mechanism underlyng this effect, however, remains to be established and perhaps is complicated by the appreciation that hypericin, a photosensitizer, is able to exert light-dependent and light-independent antitumor effects.\textsuperscript{1,4,5} In this regard, it should be noted that in all our experiments, no intentional light exposure was performed, although all work in the tissue culture hood allowed brief exposure of tumor cells to ambient light, and all tumor-bearing animals were kept under standard day–night cycles in the vivarium. Because all of the tumors were implanted subcutaneously, it is possible that these tumors were light activated, and this activation may account for the tumor’s improved activity with hypericin. An intracranial model involving hypericin is currently being developed. Perhaps more importantly, hypericin has been shown to have a high tendency to accumulate in primary and metastatic tumors and to exert antitumor activity irrespective of photoactivation.\textsuperscript{4,5}

**Conclusions**

Taken together with our finding that hypericin acts with temozolomide to enhance tumor cell killing, we conclude that the addition of hypericin could be a beneficial adjuvant to temozolomide-based chemotherapy in the treatment of GBM. Therefore, we believe that it will be worthwhile to consider this combination regimen for further evaluation in clinical trials.

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