

## Interleukin-12 and Protamine Inhibit Angiogenesis and Growth of C6 Rat Glioma: Synergistic Effects and Role of Th2 Cells

*İnterleukin-12 ve Protamin C6 Sıçan Gliomanın Anjiyogenezini ve Büyümesini İnhibe Eder: Sinerjik Etkiler ve Th2 Hücrelerinin Rolü*

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**Objectives:** This study aims to investigate the anti-tumoral, anti-angiogenic and immunologic effects of interleukin-12 (IL-12) in combination with protamine in intracranially inoculated rat C6 glioma model.

**Material and methods:** A 12-week-old 60 female Sprague-Dawley rats with a mean weight of 208±19 g were divided into six groups: C6 glioma cells were inoculated in group A; tumor in combination with recombinant IL-12 (rIL-12) 10 ng were inoculated intracranially in group B; rIL-12 10 ng intracranially with protamine 60 mg/kg subcutaneously were given to group C; protamine subcutaneously was given to group D; vehicle was given subcutaneously to group E; rIL-12 10 ng was subcutaneously given with protamine and tumor was inoculated in group F.

**Results:** Intracranial rIL-12 and protamine exerted both anti-tumoral and anti-angiogenic effects (p=0.001). Intracranial rIL-12 administration significantly diminished peripheral blood Th2 cell ratio (p=0.03) and improved survival of the rats (p=0.001). Protamine monotherapy or in combination with IL-12 showed no immunologic alteration in T helper cell population.

**Conclusion:** Recombinant IL-12 and protamine are effective against intracranially inoculated glioma, improve survival significantly as monotherapy or combination therapy and alter T helper cell cytokine secretion profile in favor of Th2 in rats.

**Key words:** Angiogenesis; C6; glioma; interleukin-12; lymphocyte; protamine; Th1 cell; Th2 cell.

**Amaç:** Bu çalışmada, interlökin-12 (IL-12) ve protaminin birlikte kullanımının intrakraniyal olarak inoküle edilmiş C6 glioma sıçan modelinde antitümöral, antianjiyojenik ve immünolojik etileri araştırıldı.

**Gereç ve yöntemler:** Altmış adet 12 haftalık ortalama 208±19 g ağırlığında Sprague-Dawley cinsi dişi sıçan altı gruba ayrıldı: Grup A'da yalnızca C6 glioma hücreleri inoküle edildi; grup B'ye tümör ile birlikte intrakraniyal olarak 10 ng rekombinant IL-12 (rIL-12) verildi; grup C'ye intrakraniyal olarak 10 ng rIL-12 ile birlikte subkutan 60 mg/kg protamin verildi; grup D'ye yalnızca subkutan protamin verildi; grup E'ye subkutan olarak yalnızca taşıyıcı verildi; grup F'ye tümör ile birlikte subkutan 10 ng rIL-12 ve protamin verildi.

**Bulgular:** İntrakraniyal olarak uygulanan rIL-12 ve protamin, hem anti-tümöral hem de anti-anjiyojenik etkiler gösterdi (p=0.001). İntrakraniyal rIL-12 uygulaması, periferik kandaki Th2 hücrelerinin oranını düşürdü (p=0.03) ve sıçanların sağkalımlarını iyileştirdi (p=0.001). Protamin, IL-12'ye ek olarak ya da tek başına T yardımcı hücre popülasyonunda ölçtüğümüz immünolojik bir değişiklik yaratmadı.

**Sonuç:** Rekombinant IL-12 ve protamin sıçanlarda intrakraniyal olarak inoküle edilen gliomaya karşı etkin olmakta, sağkalımı tek başına veya birlikte uygulanması ile anlamlı bir iyileşme sağlamakta ve periferik kandaki T yardımcı hücrelerinin sitokin salgılama profilini Th2 aleyhine değiştirmektedir.

**Anahtar sözcükler:** Anjiyogenez; C6; glioma; interlökin-12; lenfosit; protamin; Th1 hücre; Th2 hücre.

CD4<sup>+</sup> T lymphocytes play a central role in the function of the immune system by inducing B cells to produce antibodies, enhancing and maintaining the responses of the CD8<sup>+</sup> T lymphocytes, regulating macrophage function, and modulating immune responses.<sup>[1]</sup> CD4<sup>+</sup> T lymphocytes can be divided into two major groups, Th1 and Th2, according to the cytokines produced by the cells.<sup>[1,2]</sup> With regard to cytokine expression, Th1 cells produce interferon gamma (IFN- $\gamma$ ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF- $\alpha$ ), whereas Th2 cells fail to produce IFN- $\gamma$ . Their signature cytokines are IL-4, IL-5, and IL-13, and they also make TNF- $\alpha$ , with some also producing IL-9. Although initially thought to be unable to make IL-2, later results indicated that Th2 cells often produce relatively modest amounts of this cytokine signaling molecule.<sup>[1,2]</sup>

Gliomas are the most common type of brain tumors.<sup>[3]</sup> In humans, glioblastoma, a highly malignant glioma, acquires an intense vascularization.<sup>[4]</sup> The prognosis for patients with these tumors is poor, and this fact has not changed greatly in the last few decades.<sup>[5,6]</sup> Despite treatment with surgery, radiation, and chemotherapy, the two-year survival rate remains less than 20%.<sup>[3,4]</sup> Angiogenesis is a fundamental process of tumoral growth that favors the nutrition and proliferation of neoplastic cells.<sup>[7]</sup> In glioblastoma multiforme, endothelial proliferation that creates glomeruloid clusters of small vessels is one of the pathognomonic histological characteristics.<sup>[8]</sup> One of the novel therapeutic modalities in the treatment of cancer is the inhibition of angiogenesis,<sup>[9]</sup> and it has been reported that IL-12 inhibits new vessel formation.<sup>[10]</sup> In addition, it also inhibits angiogenesis indirectly through the stimulation of IFN- $\gamma$  and, at least in part, inducible protein-10 (IP-10).<sup>[11]</sup> Cockerill et al.<sup>[12]</sup> demonstrated that protamine inhibits angiogenesis and blocks endothelial, fibroblast, and platelet growth factors, and Arrieta et al.<sup>[13]</sup> showed that protamine was an effective agent against glioblastoma.

In this study, we aimed to investigate the anti-tumor potential of IL-12 and protamine administered alone and in combination and also examine the role of the pathways of angiogenesis and anti-tumor immunity and their effects.

## MATERIAL AND METHODS

### Tissue culture and cell line

Institutional review board approval was obtained before commencing this study. Initially, the C6 glioma cells<sup>[14]</sup> obtained from the American Type Culture Tissue Collection (Rockville, Maryland, USA) were cultured at 37 °C under sterile conditions in an F12-

cell culture medium supplemented with 15% fetal calf serum, 0.2 mM glutamine, 50 mg/mL neomycin, and 100 mg/mL streptomycin. To obtain a large source of C6 cells, 2x10<sup>6</sup> cultured C6 cells were intracranially implanted. In this procedure, the glioma cells were isolated via 2 ml trypsin ethylenediaminetetraacetic acid (EDTA) solution C (Biological Industries, Kibbutz Beit Haemek, Israel) and centrifuged after the addition of 2 cc Dulbecco's modified Eagle's medium (Merck, Darmstadt, Germany) along with a 10% fetal calf serum mixture at 1x10<sup>3</sup> rpm for three minutes. After removing the supernatant, the pellet was resuspended with 2 cc of the same medium in a cell containing 2x10<sup>8</sup> cells/mL. The viability of the cells was checked using a trypan blue exclusion test every hour. Next, 12-week-old female virgin Wistar rats, which weighed 208±19 gr, were anesthetized with 45 mg/ml of 10% ketamine and 8.6 mg/ml of 2% xylazine. The rats were stabilized with a stereotaxis apparatus (Trent Welles Inc., South Gate, Ca, USA), and after antisepsis was completed with a polyvinylpyrrolidone (PVP) solution, a 1 cm skin incision was made at the mid-sagittal line. A burr hole (1.5 mm wide) was then created with a dental drill on the right side that measured 3 mm from lateral to midline and 2 mm proximal to the bregma. Afterward, a Hamilton syringe featuring a 27 gauge needle fastened to the manipulation arm of the stereotaxic apparatus was used to inject the C6 glioma cells. It was then further inserted to the center of the right caudate nucleus, located 5 mm from the surface of the brain. In 10 minutes, we injected a total of 10 microliters of cells. The needle remained in place for five minutes and was then removed very gently over a two-minute period. Next, the incision area was washed with saline, and the burr hole was closed with bone wax. The scalp was then closed using 5-0 polyglactin sutures (Ethicon, Somerville, NJ, USA).

In two groups of rats (n=10), a suspension of 1x10<sup>7</sup> cells in 500  $\mu$ l of saline solution was then inoculated subcutaneously into the left thigh of the 12-week-old female Wistar rats. A subcutaneous tumor developed in 90% of these animals that measured 2 cm in diameter on average 20 days after the cell implantation.

### Administration of protamine, IL-12 and protamine-IL-12

The rats were divided six groups. Group A (n=10) was used as the tumor control group, and 2x10<sup>6</sup> cultured C6 cells were intracranially implanted in these rats. To study the effect of IL-12 on glioma, the animals from group B (n=10) were treated with 10 ng of IL-12 (Merck, Darmstadt, Germany) intracranially during the glioma implantation (2x10<sup>6</sup> cells) (administered with the tumor). To study the effect of IL-12 along with protamine, the

rats from group C (n=10) were given daily doses of 10 ng of IL-12 intracranially (with  $2 \times 10^6$  glioma cells) along with 60 mg/kg subcutaneous protamine (Sigma-Aldrich, Munich, Germany) for five days. The rats in group D (n=10) were treated with 60 mg/kg subcutaneous protamine (Sigma-Aldrich, Munich, Germany) for five days after the glioma implantation ( $2 \times 10^6$  cells). Group E (n=10) was used as a control, so the animals were injected subcutaneously with 0.5 ml saline solution every day for five days. Finally, group F (n=10) was treated with 10 ng subcutaneous IL-12 and 60 mg/kg protamine daily for five days.

All of the rats were monitored daily for any focal motor deficits, bradykinesia, or other major neurological symptoms. Between the 12 and 16<sup>th</sup> days, most of the rats injected with an intracranial tumor developed left hemiparesis and/or bradykinesia. The animals were maintained according to the guidelines established by the Institutional Animal Care and Use Committee of our institution, and the experiments were conducted accordingly. On the 30<sup>th</sup> day of the experiment, all of the rats were decapitated under anesthesia for further evaluation.

### Histopathological evaluation

The brains fixed in 10% buffered formalin were dissected on 10 mm coronal slides using the injection hole as the origin. Glass slides measuring 4 microns in thickness were prepared, and from these, histology sections were stained utilizing the conventional hematoxylin-eosin (H-E) technique for routine analysis. In addition, monoclonal marking for CD34 expression was performed via streptavidin immunohistochemistry, and the number of intra- and peri-tumoral vessels and glomeruloid microendothelial proliferations were counted with five high power field (HPF) area ( $0.238 \text{ mm}^2$ ). According to this data, two indices were calculated: i1 (the number of intra-tumoral vessels/total tumor area) and i2 (the number of peri-tumoral vessels/the number of intra-tumoral vessels).

### Intracellular cytokine expression

For the detection of intracytoplasmic cytokine expression (the identification of Th1 and Th2 cells), the peripheral blood mononuclear cells obtained from the tail vein, which were stimulated with immobilized anti-human-CD3 monoclonal antibody (mAb) (Advanced Medical Science Co., Ltd., Bangkok, Thailand) for six hours in brefeldin A (Wako Pure Chemical Industries, Ltd., Osaka, Japan), were first stained with anti-human-CD4-fluorescein isothiocyanate (FITC) (Advanced Medical Science Co., Ltd., Bangkok, Thailand). This was fixed with 1% paraformaldehyde and treated with a

permeabilizing solution (50 mM sodium chloride (NaCl), 5 mM EDTA, 0.02 sodium azide (NaN<sub>3</sub>), 1% Triton X-100, pH 7.5). The fixed cells were then stained with anti-human-CD4-phycoerythrin (PE) (Advanced Medical Science Co., Ltd., Bangkok Thailand) and anti-human interferon (IFN)-gamma-FITC (Advanced Medical Science Co., Ltd., Bangkok, Thailand) for 45 minutes, and the percentage of cells expressing cytoplasmic IL-4 or IFN- $\gamma$  was determined using the BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

### Statistical analysis

Survival was evaluated via Kaplan-Meier cumulative hazard plots and a Wilcoxon analysis, with the differences being considered significant when  $p$  was  $<0.05$ .

## RESULTS

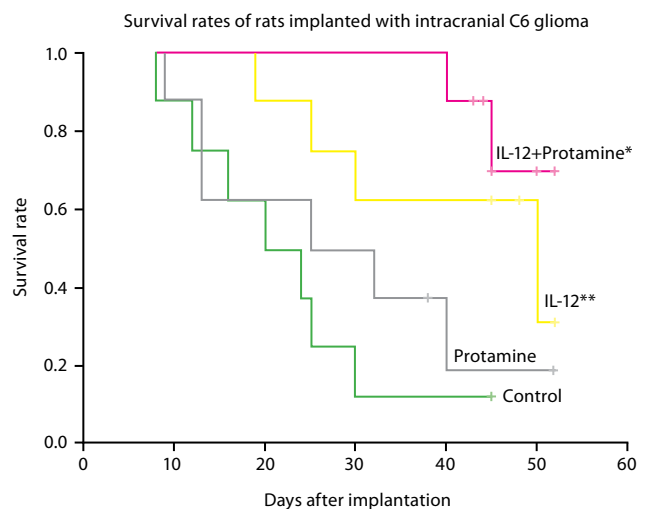
### Survival

The survival rates after the intracranial implantation of the C6 cells into the right caudate of the adult Wistar rats are shown in Figure 1. The subcutaneously-injected C6 tumors became palpable after 10 days and grew to an average maximum size of  $11.2 \pm 2.1 \text{ mm}$ .

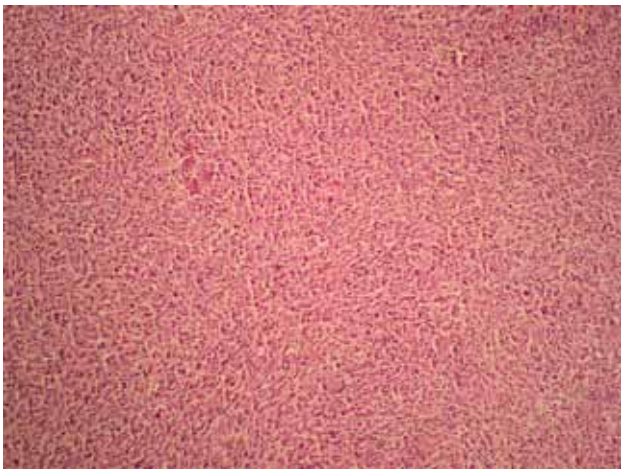
Groups A and C had statistically significantly better survival rates than group D ( $p=0.03$ ) and group E ( $p=0.001$ ) (Figure 1). Furthermore, the rats in group B had higher survival rates than group E ( $p=0.02$ ). Figure 2 shows the microscopic appearance of the C6 glioma tissue extracted from groups B and D.

### Vascularization

The vascularization was localized in the transition zone between the tumor and the brain parenchyma.



**Figure 1.** Survival curves of rats. \*  $p=0.001$ ; \*\*  $p=0.03$  compared with the control group.



**Figure 2.** A histological section of the tumor of a rat that was administered IL-12 and protamine. (H-E x 400) No microvessels were identified.

In groups B and C, there tended to be more tumoral lymphomonocytic infiltration groups D and E. In addition, the vascularization was strongly inhibited in groups B and C (Table 1). When IL-12 was given with protamine, the anti-vascularity effect seemed to be synergistic, and the intra-tumoral vascularization in group C was lower than in group D ( $p < 0.04$ ). Moreover, peri-tumoral vascularization was prominently diminished in groups B and C compared with group E ( $p = 0.02$  and  $p = 0.001$ , respectively) and group D ( $p = 0.02$  and  $p = 0.01$ , respectively) (Table 1). The vascularization indices were not evaluated in group A since these indices are not reliable when evaluating subcutaneous tumors.

**T lymphocyte subgroups**

The CD3<sup>+</sup> T lymphocyte rates (percentage of all lymphocytes) were between 50 and 70%, and there

**TABLE 1**

Intra-tumoral (i1) and peri-tumoral (i2) vascularization in rats implanted intracranially with C6 glioma

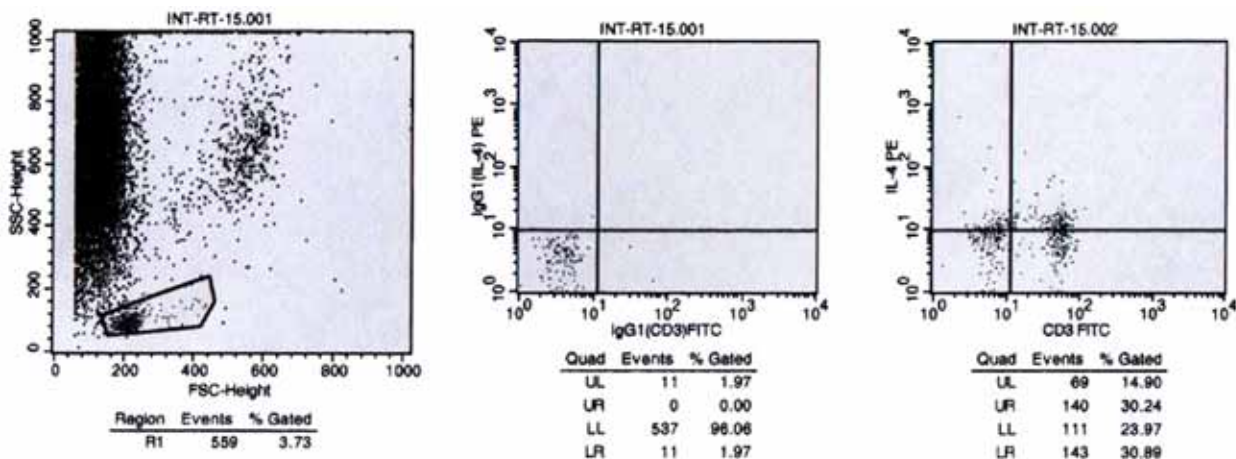
Indices	Control	IL-12	IL-12 + protamine	Protamine
i1	85	79	7.2*	57
i2	19	0.9**	0.6§	14

\*  $p < 0.001$  (compared to the control group); \*\*  $p < 0.001$  (compared to the control group); §  $p < 0.001$  (compared to the control group).

were no statistically significant differences in the T lymphocyte rates themselves. The Th1 and Th2 cells were reliably evaluated using flow cytometry (Figure 3), and there was a statistically significant decrease in the Th2 cells in groups B and C ( $p = 0.03$ ) (Table 2). Furthermore, the Th1 cells seemed to be unaffected by IL-12 and/or protamine administration, but protamine seemed to boost the number of Th2 cells in group D ( $p = 0.003$ ) (Table 2).

**DISCUSSION**

Many brain tumors, particularly gliomas, display an invasive phenotype when tumor cells infiltrate the surrounding tissues. For this reason, it is difficult to cure these tumors. Deep tumors that are in non-eloquent brain areas can be easily accessed and removed, whereas superficial tumors may not be easily resected because of their location within extremely eloquent brain tissue.<sup>[3,4,14]</sup> Chemotherapy for brain tumors involves many of the same problems as it does with systemic cancer, including a lack of specificity, an intrinsic or developing cellular resistance, an intolerance of normal tissue to drug toxicity, synergistic toxicity between chemotherapy and radiation therapy, and systemic toxicity.<sup>[14]</sup> Therefore, surgical therapy, chemotherapy, and radiation therapy have provided unsatisfactory results for patients with glioma.<sup>[14]</sup>



**Figure 3.** The Th2 cells were identified as CD3<sup>+</sup>CD4<sup>+</sup>IL-4<sup>+</sup> cells.

TABLE 2

Lymphocyte subgroup ratios in the control and study groups

Lymphocyte subgroups	Group A	Group B	Group C	Group D	Group E	Group F
Th1 cells (%)	59.9±5.6	49.2±5.7	49.2±4.3	39.8±3.3	50.8±9.4	50.8±9.8
Th2 cells (%)	9.1±7.1	5.8*±4.0	5.5*±2.8	17**±3.8	9.4±3.4	9.4±3.4

\* p=0.03 (compared with groups A, E, and F); \*\* p=0.003 (compared with groups A, B, E, and F)

Studies over the past 30 years have provided valuable insights into the angiogenic process and its role in cancer biology, with over 17,000 papers published on the topic. Additionally, the cloning of vascular endothelial growth factor (VEGF) in 1989 was a major milestone in our understanding of tumor angiogenesis.<sup>[15]</sup> Interleukin-12 is a heterodimeric cytokine that plays a central role in promoting Th1 responses and cell-mediated immunity,<sup>[1,2,14]</sup> and recombinant (rIL-12) has had striking therapeutic effects in a number of rodent tumor models, including C6 glioma.<sup>[13,15,16]</sup> Our study demonstrated that intracranial, intra-tumoral rIL-12 possesses significant single-agent anti-tumoral activity and that a treatment regimen consisting of IL-12 administered in combination with protamine is superior to either agent alone. In addition, this effect was associated with anti-angiogenic activity and the induction of Th1 cells. Th2 cells were also diminished in the rats that were given IL-12. These findings suggest that IL-12 has an anti-tumoral role and targets tumor angiogenesis in rat C6 glioma models. We found that peri-tumoral vascularization was prominently diminished in group C compared with groups D and E, but we were not able to conclude whether these agents use the same pathways or different ones against angiogenesis. The survival rates in group C were the highest, and almost no vascular structure was discovered in the extracted tumor tissues from the rats in that group. However, it must be emphasized that our findings need further confirmation anomalies in order to determine whether they were due to the extreme anti-angiogenic effect of these two agents or if they resulted from the sensitivity of our histopathological evaluation of angiogenesis. Other immunohistochemical staining with as anti-VEGF or anti-CD34 antibodies,<sup>[9,10]</sup> could be used for this purpose.

Yao et al.<sup>[17]</sup> found that NK cell function was required for IL-12 to be an inhibitor of angiogenesis, and they proposed that IL-12 inhibits angiogenesis indirectly through the stimulation of IFN- $\gamma$  and IP-10. On the other hand, Cockerill et al.<sup>[12]</sup> determined that protamine directly inhibits angiogenesis.

Interestingly, we found that intracranial rIL-12 administration caused a marked increase in peripheral blood Th1 cells but a decrease in Th2 cells, and to our

knowledge, no study has demonstrated any connection between cranial IL-12 and the peripheral blood T cell subsets and/or cytokine responses. Recently, Tanriover et al.<sup>[18]</sup> found that the systemic administration of IL-12 in a C6 glioma model in rats prolonged their survival most likely by stimulating the cellular immune infiltration, but they were not able to test this hypothesis. Nevertheless, a number of studies have proven that there is a possible link between IL-12 and anti-tumor immunity as well as Th1 cells and the brain as an immune privileged organ.<sup>[19,20]</sup>

DiMeco et al.<sup>[21]</sup> demonstrated that local delivery of IL-12 into the rat brain by genetically engineered cells significantly prolongs the survival time in animals with intracranial malignant glioma. However, they did not assess any potential changes in the local or systemic immune response. Aloisi<sup>[22]</sup> was the first to describe that microglia produces IL-12, which mainly promotes the development of Th1 cells. However, there is no evidence to indicate that glioma cells produce IL-12 or any of the Th1-inducing cytokines.

Arrieta et al.<sup>[13]</sup> first demonstrated that protamine inhibits angiogenesis and C6 rat glioma, and our findings verified their conclusions. In addition, we found that protamine and IL-12 synergistically inhibit angiogenesis, thereby prolonging survival. We also determined that protamine can cause Th2 cell stimulation. However, this effect did not seem to neutralize the anti-tumoral and Th2-inhibiting effect of intracranially injected IL-12. To our knowledge, there have been no studies in the literature that have demonstrated any immunological properties associated with protamine, but this still needs to be thoroughly investigated.

In our study, we aimed to combine the anti-tumoral and anti-angiogenic properties of IL-12 and the anti-angiogenesis effect of protamine in rats. Our findings suggest that these two agents could form a useful combination for the treatment of glioma. However, human studies are needed to confirm these findings.

#### Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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