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## Preclinical report

# Bryostatin 1 induces differentiation and potentiates the antitumor effect of Auristatin PE in a human pancreatic tumor (PANC-1) xenograft model

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**Pancreatic cancer has the worst prognosis of all cancers with a dismal 5-year survival rate. Hence, there is a tremendous need for development of new and effective therapy for this tumor. In an earlier study we reported a potent antitumor activity of Auristatin PE (AuriPE) against pancreatic tumor. In addition, we have also reported that bryostatin 1 (bryo1) induces differentiation of leukemia cells, but the effect of bryo1 has not been investigated in pancreatic tumors. This is the first report where we demonstrate that bryo1 induces differentiation and potentiates the antitumor effect of AuriPE in a human pancreatic tumor (PANC-1) xenograft model. A xenograft model was established by injecting the PANC-1 cells s.c. in severe combined immune deficient (SCID) mice. After development of the s.c. tumors, tumors were dissected and small fragments were transplanted *in vivo* to new SCID mice, with a success rate of 100% and a doubling time of 4.8 days. The SCID mouse xenograft model was used to test the *in vivo* differentiation effect of bryo1 and its efficacy when given alone or in combination with AuriPE. Sections from paraffin-embedded tumors excised from untreated (control) SCID mice revealed typical poorly differentiated adenocarcinoma of the pancreas. Interestingly, sections of s.c. tumors taken from bryo1-treated mice revealed carcinomas that were much lower grade and less aggressive, and displayed prominent squamous and glandular differentiation. In this study, the tumor growth inhibition (T/C), activity score and cure rate for bryo1, AuriPE and bryo1+AuriPE were 80%, (+) and 0/4; 0.0%, (++++) and 3/5; and 0.0%, (++++) and 3/4, respectively. Mice treated with either AuriPE or bryo1+AuriPE were free of tumors for more than 150 days and were considered cured. The use of bryo1 as a novel differentiating agent and its combination with AuriPE should be further explored for the treatment of adenocarcinoma of the pancreas. [© 2001 Lippincott Williams & Wilkins.]**

*Key words*: Auristatin PE, bryostatin 1, PANC-1, pancreatic tumor line, SCID mice, xenograft.

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## Introduction

Pancreatic cancer became the fourth leading cause of cancer-related deaths (preceded by lung, breast/prostate and colorectal cancers) among both male and female adults in the US with an estimated 28 200 deaths in 2000.[1] Pancreatic cancer is commonly diagnosed late in its natural history and as a result the 5-year survival rate is less than 1%. Its etiology is largely unknown, but may be related to either environmental or acquired factors and no effective method of early diagnosis or treatment is presently available. The current treatment of localized non-metastatic adenocarcinoma of the pancreas is based on the surgical procedure (pancreatoduodenectomy) first described in 1935 by Whipple *et al.*[2] The interval from the onset of symptoms until the patient's death is usually between 5 and 9 months. Thus, the early diagnosis and management of patients with pancreatic tumor presents a real challenge for clinical and laboratory investigators.

Pancreatic cancer is highly resistant to conventional therapies; therefore, treatment methodologies of surgery, radiation therapy and chemotherapy used singly or in combination have made little or no impact on this disease. However, basic scientific research has improved our understanding of the biology of pancreatic cancer, resulting in the identification of a number of targets that could be exploited in future

clinical trials. Since conventional therapies in pancreatic cancer have largely failed, development of new treatment regimens and innovative therapeutic strategies becomes a necessity.

Among new treatment modalities is the use of biological agents, such as Bryostatin 1 (bryo1) and Auristatin PE (AuriPE). Bryo1 is a macrocyclic lactone obtained from a marine organism *Bugula neritina* which exhibits various biological properties such as antitumor and immunomodulating activity against murine[3] and human[5–7] tumors. Using severe combined immunodeficiency (SCID) mouse xenograft models, we have also demonstrated antineoplastic activity for bryo1 alone and an ability to potentiate activity of certain cytotoxic chemotherapy agents.[8] Bryo1 entered phase I clinical trials in the US in 1994. Using a 72-h continuous i.v. infusion every 2 weeks, the maximum tolerated dose (MTD) in humans was found to be 120 $\mu g/m^2$.[9]

AuriPE is a structural analog of dolastatin 10 developed by replacing a dolaphenine unit with phenethylamine (PE).[10] Dolastatin 10 was isolated from the sea hare *Dolabella auricularia*. It is a linear tetrapeptide linked to a complex primary amine and interacts with tubulin to inhibit microtubule polymerization.[11] We have previously demonstrated the antitumor activity of AuriPE against pancreatic tumor cells; however, no studies are available exploiting the utility of Bryo1 alone or in combination with AuriPE as antitumor therapy in the pre-clinical setting for pancreatic tumor. Hence, pre-clinical evaluation of these agents for pancreatic adenocarcinoma requires the development of an appropriate animal model of human pancreatic cancer.

In this study, we examined whether Bryo1 induces differentiation and potentiates the antitumor effect of AuriPE *in vivo* using a human pancreatic tumor (PANC-1) xenograft model. Our results clearly demonstrate, for the first time, that bryo1 act as a potent inducer of tumor differentiation into less aggressive forms and significantly enhances the antitumor effect of AuriPE against human pancreatic tumor xenografts.

## Materials and methods

### PANC-1 cell line

PANC-1, a well-established pancreatic adenocarcinoma cell line, purchased from the ATCC (Rockville, MD) was used in this study. Cells were grown in DMEM:F12 (1:2) medium supplemented with 5% FBS, 15 mM HEPES, 2 $\mu$g/ml insulin, 5 $\mu$g/ml transferrin, 40 ng/ml hydrocortisone and 10 ng/ml epidermal growth factor at 37°C in a humidified 5% $CO_2$ atmosphere.

### Bryo1 and AuriPE

Bryo1, a macrocyclic lactone and a potent activator of protein kinase C, was extracted and purified from the marine animal *B. neritin*a.[7] It has antitumor and immune modulating activity on a number of malignancies.[4–7] AuriPE is a small peptide isolated from the marine sea hare *D. auricularia*. They are potent antineoplastic agents that cause cells to arrest in metaphase by binding to microtubule components in the cells, particularly to tubulin.[10,11]

### The PANC-1 xenografts

Four-week-old female ICR SCID mice were obtained from Taconic Laboratory (Germantown, NY). Upon arrival, the mice were kept under specific pathogen-free environments in the animal facility of Karmanos Cancer Institute, Wayne State University. After a period of adaptation, each mouse received $10^7$ PANC-1 cells (in serum-free RPMI 1640) s.c. in each flank area. When s.c. tumors developed, mice were sacrificed, and tumors were dissected and mechanically cut into small fragments (about 30 mg). For the subsequent drugefficacy trials, small fragments of the PANC-1 xenograft were transplanted s.c. in to similarly conditioned animals, using a 12 gauge trocar. Mice were checked 3 times a week for tumor development. Once palpable tumors developed, groups of four animals were removed randomly for different treatments and a control category. Each group received injections at the MTD. Bryo1 (75 $\mu$g/kg; i.p.), AuriPE (1.5 mg/kg; i.v.) and bryo1+AuriPE (75 $\mu$g/kg; i.p. +1.5 mg/kg; i.v.).[12] Changes in weight and side effects of the drugs were recorded. Animals were euthanized when their total tumor burden reached 1500 mg (8% of body weight) to avoid discomfort.

The end points for assessing antitumor activity were according to standard procedures used in our laboratories[12,13] and were as follows: (i) tumor weight (mg)=$(A \times B^2)/2$, where $A$ and $B$ are the tumor length and width (in mm), respectively; (ii) tumor growth inhibition (T/C) is the median tumor weight in the treated group (T) when the median tumor weight in the control group (C) reached approximately 730 mg; (iii) tumor growth delay (T–C) where T is the median time (in days) required for the treatment group tumors to reach 730 mg and C is the median time (in days) for the control group tumors to reach same weight; and (iv) tumor cell kill total $(\log_{10})$=(T–C)/(3.32)($T_d$), where $T_d$ is tumor doubling time. All studies involving mice were performed under Institutional Review Board approved protocol. A rating score of (+++) is considered active and (++++) is highly active. A rating

score of (+++) is needed to induce partial and (++++) to induce complete tumor regressions, (++) indicates marginal activity, and (+) is not active.

### Calculation of $T_d$ in mice

Tumor weights in SCID mice were plotted against time on a semi-log sheet. The growth pattern was close to an S-shape. $T_d$ is the time (in days) required for a tumor to double its weight during the exponential growth phase.

### *In vivo* differentiation of PANC-1 tumor

The SCID mouse xenograft model was also used to test the differentiating effect of bryo1 on PANC-1 cells. SCID mice treated with bryo1 (75 $\mu$g/kg; i.p.) were compared with untreated mice (control). Tumors were excised, fixed in 10% buffered formalin and paraffin embedded. Then, 5-$\mu$m tissue sections were used for HE staining and for immunohistochemistry by methods described previously.[14,15]

## Results

### Bryo1 induces differentiation of the PANC-1 tumor in the *in vivo* xenograft model

Sections from paraffin-embedded tumors excised from untreated SCID mice revealed typical poorly differentiated adenocarcinoma of the pancreas. Interestingly, sections of s.c. tumors taken from bryo1-treated mice revealed much lower-grade carcinomas, and, in addition, displayed prominent squamous and glandular differentiation (Figure 1). One very important effect of bryo1 that we have identified in our pilot study is that in some of the xenograft tumors treated with this agent, the tumor cells had transformed into a much-differentiated form of pancreatic carcinoma. In fact, in some instances the tumors displayed a cystic and papillary pattern in contrast to the solid anaplastic and pleomorphic patterns in untreated cases or those treated with other agents. These cystic and papillary patterns are the characteristic finding of low-grade pancreatic neoplasia (intraductal papillary mucinous neoplasms or mucinous cystic neoplasms which have a better clinical course than that of ordinary pancreas cancer; 5-year survival of 70 versus 2%, respectively). This is the first time we observed an *in vivo* differentiating effect of bryo1 in a pancreatic tumor, suggesting that the use of bryo1+AuriPE should be further explored in the treatment of adenocarcinoma of the pancreas.

### Xenografts and *in vivo* drug efficacy of bryo1 and AuriPE in the xenograft tumor

When PANC-1 was transplanted in SCID mice, the take rate was 100%. Drug efficacy trials were conducted on animals with palpable tumors. Table 1 shows the activity of bryo1, AuriPE and bryo1+AuriPE against PANC-1 *in vivo*. In this study, the tumor growth inhibition (T/C), activity score and cure rate for bryo1, AuriPE and bryo1+AuriPE were 80%, (+) and 0/4; 0.0%, (++++) and 3/5; and 0.0%, (++++) and 3/4, respectively. Mice treated with either AuriPE or bryo1+AuriPE were free of tumors for more than 150 days and were considered cured.

When tumor responses are determined by the T/C value, bryo1 alone (T/C=80%) is considered inactive against PANC-1 tumor (a T/C value of 42% or less is considered significant antitumor activity by the Drug Evaluation Branch of the Division of Cancer Treatment, NCI[13]). The bryo1+AuriPE combination showed excellent activity by usual clinical criteria with an activity score of (++++) and three out of four treated mice were tumor free.

## Discussion

Pancreatic cancer is the fourth leading cause of cancer-related mortality in males in the US. It remains generally incurable by available treatment modalities. This is the first report where we demonstrate that bryo1 induces differentiation of the human pancreatic tumor PANC-1 in a SCID xenograft model. Moreover, when given in combination, it potentiated the antitumor activity of AuriPE (a new microtubule polymerization inhibitor) and resulted in cure rate.

Recently, anticancer drug discovery efforts have focused on natural sources such as plants and marine animals. The results of these efforts have revealed several novel compounds like the bryostatins. Bryo1 was isolated from an Eastern Pacific colonial marine filter feeder *B. neritina* in 1982.[7] Bryo1 is a natural, macrocyclic lactone and a protein kinase C activator/deactivator, and has differentiation activity against murine and human lymphoma and leukemia cells.[47] A xenograft model was established by injecting the PANC-1 cells s.c. in SCID mice. After development of the s.c. tumors, they were dissected and small fragments were transplanted *in vivo* to new SCID mice, with a success rate of 100% and a doubling time of 4.8 days. One very important effect of bryo1 that we have identified in this study is that in xenograft tumors treated with 75 $\mu$g/kg bryo1, i.p., the tumor cells had transformed into a much-differentiated form of pan-

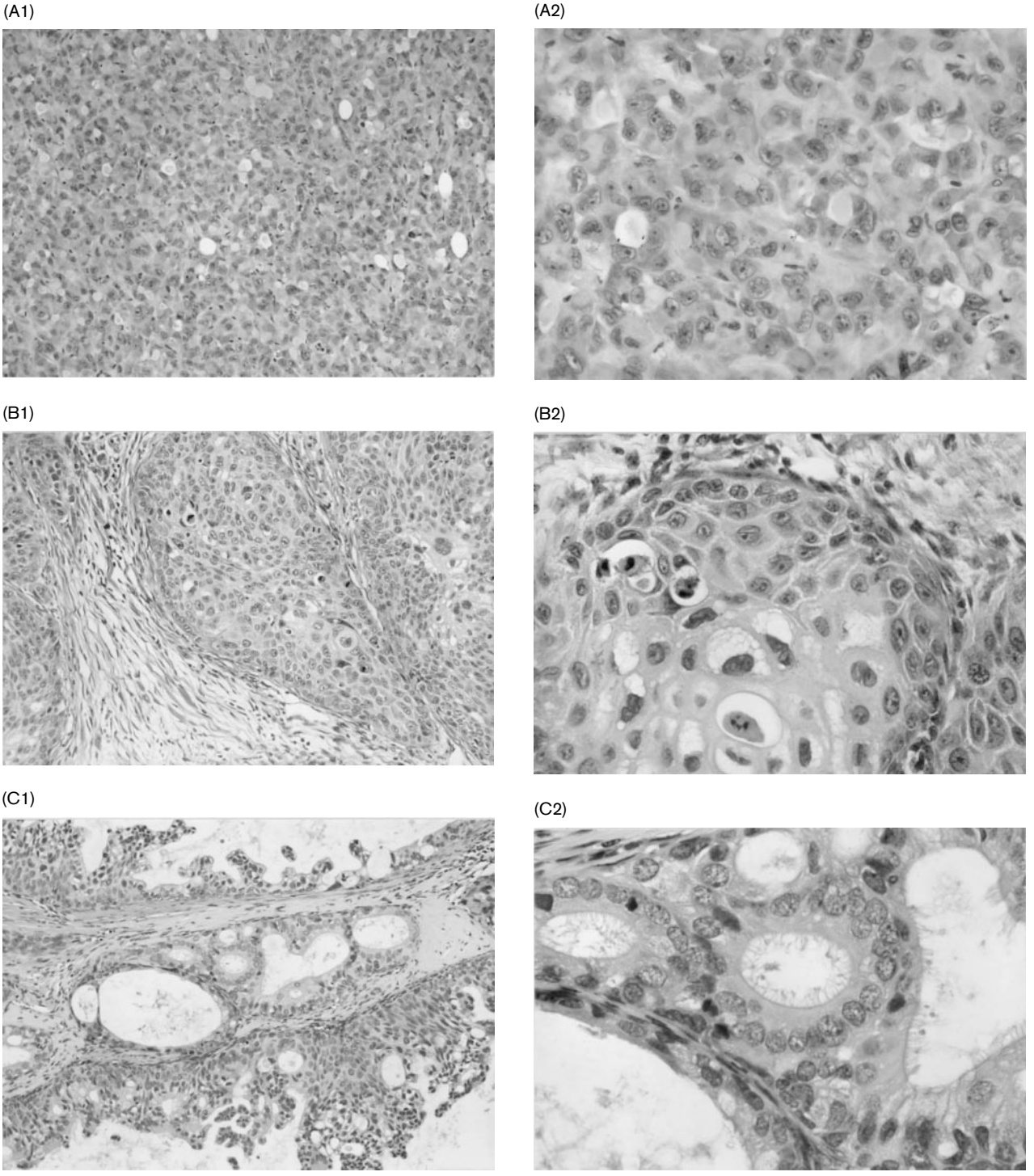


**Figure 1.** Photomicrographs showing H&E staining demonstrate typical ductal adenocarcinoma of the pancreas. Tissue sections (5 $\mu$m) taken from bryo1-treated mice revealed carcinoma with squamous and glandular differentiation. (A) Undifferentiated carcinoma form an untreated xenograft tumor. A diffuse sheet of malignant cells with no specific patterns (A1, low power). Cytomorphologically the tumor cells are large with marked pleomorphism (A2) and high nucleocytoplasmic ratio demonstrating the irregular nuclear contours with prominent nucleoli, typical features of poorly differentiated adenocarcinoma of the pancreas. (B) Carcinoma with squamous differentiation in a xenograft tumor treated with bryo1. The tumor cells formed well-defined nests separated by stroma (B1, low power). In high-power photomicrograph (B2), the cells at the periphery of these nests are well polarized and have basaloid features (high neucleocytoplasmic ratio and minimal cytoplasm). Towards the center of the nests, they acquire abundant pink cytoplasm, i.e. squamous differentiation. The desmosomes are prominent

creatic carcinoma (Figure 1). In fact, in some instances the tumors displayed a cystic and papillary pattern in contrast to the solid anaplastic and pleomorphic patterns in untreated cases or those treated with other agents. These cystic and papillary patterns are characteristic features of some low-grade forms of 'pancreas cancer' that have a starkly better clinical course (5-year survival greater than 70%) and biologic behavior. In fact, how these lower-grade neoplasia relate to ordinary ductal adenocarcinoma, which has a 5-year survival less than 5%, is one of the most important dilemmas of 'pancreas cancer'. The answers to why pancreas cancer has such a terrible prognosis may partly lie in the differences between these tumors.

Genetic changes leading to imbalance between cell division and differentiation are associated with growth advantage for neoplastic cells. Based on this view standard therapy has concentrated on cell kill utilizing a variety of chemotherapy agents and radiation therapy. However, activation of a differentiation pathway may be a way to reverse neoplasia or change its genetic characteristics and achieve better therapeutic results. The validity of such rationale and its clinical applicability has been demonstrated by using all-*trans*-retinoic acid (ATRA) in acute promyelocytic leukemia.[16] ATRA given orally to patients with APL was found to induce differentiation of the leukemic promyelocytes to the terminal stage of neutrophil. Likewise, bryo1 has been documented to have antineoplastic properties against a number of murine cell lines including B16 melanoma, M5076 reticulum cell sarcoma and the L10A B cell lymphoma.[3,7] Bryo1 was also shown to be responsible for the induction of specific tumor types to differentiate. It induces differentiation in acute lymphoblastic leukemia[17] and chronic lymphocytic leukemia (CLL),[18,19] and non-Hodgkin's lymphoma.[6]

The SCID mouse xenograft model was used to test the efficacy of bryo1 and its combination with AuriPE. When tumor responses were determined by the T/C value, bryo1 alone (T/C=80%) was considered inactive against PANC-1 tumor (a T/C value of 42% or less is considered significant antitumor activity by the Drug Evaluation Branch of the Division of Cancer Treatment, NCI[13]). However, the bryo1+AuriPE combination showed excellent activity by usual clinical criteria (three of four mice cured, activity score=++++). It should be noted, however, that while the response criteria we adopted in our animal studies are standard, they do not necessarily directly translate to partial or complete response criteria in human patients. We have adopted a scoring system of drug activity that correlates better with response criteria in humans.[20] In this study, the antitumor activity is considered highly active (++++) when the $\log_{10}$ kill (net) is greater than 2.0. An activity rating score of (++++) is needed to effect complete tumor regression clinically. A score of [+++; (net 0.8–2.0)] is needed to effect partial tumor regression, a score of [++; (net 0.550.8)] is marginal and [+; (net $<0.5$)] is not considered active by usual clinical criteria.

**Table 1.** *In vivo* dose, schedule and antitumor activity of byro1, AuriPE and bryol1+AuriPE against PANC-1-bearing SCID xenografts

| Agent | Dose[a] | Route | No. of animals | No. of injections | T/C (%) | Cures | Activity score[b] |
|---|---|---|---|---|---|---|---|
| Diluent | 0.0 mg/kg | i.v. | 4[c] | 3 | 100 | 0.0 | – |
| Bryo1 | 75 $\mu$g/kg | i.p. | 4 | 3 | 80 | 0.0 | + |
| AuriPE | 1.5 mg/kg | i.v. | 5 | 3 | 0.0 | 3/5 | ++++ |
| Byro1+AuriPE | 75 $\mu$g/kg+1.5 mg/kg | i.p. i.v. | 4 | 3 | 0.0 | 3/4 | ++++ |

[a]Doses were determined based on previous experiments with these drugs.
[b]Rating score of +++ (active) or ++++ (highly active) is needed to effect partial or complete tumor regressions, ++ (marginal activity), and + is not active.
[c]SCID mice with bilateral tumors (two tumors per mouse).

at the intracellular spaces (B2), another characteristic manifestation of squamous differentiation. Furthermore, the microvesicular cytoplasm in some of these cells may be indicative of early sebaceous differentiation. (C) Carcinoma with glandular differentiation (adenocarcinoma) in a xenograft tumor treated with bryo1. The tumor is characterized by a cystic, glandular and papillary growth pattern typical of adenocarcinoma (C1, low power). On high-power examination (C2), the tumor cells display features of well-differentiated adenocarcinoma: round cells that are well polarized around the lumen. The presence of cilia lining the apical surface of the cytoplasm is another indication of how well this tumor is differentiated.

We have previously demonstrated that bryo1 when given in combination with AuriPE produces a synergistic effect *in vitro* as well as in SCID mice bearing CLL *in vivo* where the animals were free of tumors.[20] In one of our experiments, administration of AuriPE alone, using the same dose schedule against KCI-MOH1 pancreatic xenograft, showed modest activity.[21] However, when given in combination with gemcitabine, this resulted in a synergistic effect with two out of seven mice remaining tumor free. In a second study, using human pancreatic adenocarcinoma (HPAC) cells in an orthotopic model, we also found that the AuriPE+gemciatbine combination was an effective regimen.[22]

In summary, we conclude that (i) this is the first study illustrating that bryo1 induces differentiation of the human pancreatic tumor PANC-1 *in vivo* in a SCID xenograft model, and (ii) when given in combination, bryo1 potentiated the antitumor activity of AuriPE. The results of this preliminary study suggest that these agents should be explored clinically for the treatment of pancreatic cancer.

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