The Matrix Metalloproteinase Inhibitor Batimastat (BB-94) Retards Human Breast Cancer Solid Tumor Growth but not Ascites Formation in Nude Mice

ABSTRACT

Matrix metalloproteinases (MMPs) are thought to play a significant role in tumor invasion and metastasis as well as angiogenesis. Batimastat, also known as BB-94, acts as an inhibitor of metalloproteinase activity by binding the zinc ion in the active site of MMPs. In our study, the hormon-independent MDA435/LCC6 human breast cancer cell line was used to seed solid tumors s.c. into the region of the mammary fat pad in athymic nude mice. Mice were treated with 50 mg/kg batimastat i.p. Tumor volume measurements showed a statistically significant decrease in tumor size between batimastat-treated and control animals. In contrast, we also used the same MDA435/LCC6 cell line to propagate a malignant ascites in nude mice, which yielded a very different response to batimastat. Batimastat, in previously published literature, had been shown to prolong the life of mice bearing ovarian ascites tumors. Treatment with batimastat in our ascites model produced no increase in survival or significant suppression of ascites formation. However, treated animals showed dramatic tumor cell consolidation and less dispersed ascites cells compared with control animals. Two potential targets of batimastat, gelatinase A and B (MMP-2 and -9, respectively), were examined in both tumor sites. These metalloproteinases were present in both solid tumor and ascites fluid and in both cases were host tissue-specific role of MMPs in breast cancer tumor progression.

INTRODUCTION

MMPs are believed to play a necessary role in tumor invasion and metastasis, as well as new blood vessel formation, a process called angiogenesis (see reviews, Refs. 1–3). MMPs may be produced either in the tumor or its underlying stroma and may act by degrading the extracellular matrix, the barrier between tissue compartments, thereby allowing tumor cells access to previously restricted areas. Breakdown of the extracellular matrix is also necessary to allow tumor cells access to lymph and blood vessels to metastasize, as well as to allow new blood vessels to invade through tissue to form new vasculature for tumor nourishment and oxygenation. The MMPs constitute a family of more than a dozen secreted or membrane-bound proteins that cleave extracellular matrix components such as the collagens, gelatin, fibronectin, laminin, and elastin. The zymogen form is converted to an active form by removal of an amino-terminal domain. All of the MMPs use a zinc-binding site in the catalytic domain.

A class of endogenous inhibitors, called TIMPs act to inhibit MMP precursors as well as the active proteins (reviewed in Ref. 2). The extent of matrix degradation depends on the balance between active and inactive MMPs and also possibly on the ratio of MMPs to their inhibitors. Studies have shown that overexpression of TIMPs in the tumor setting can inhibit tumor invasion and metastasis (4, 5). Similarly, inhibition of TIMP by antisense RNA enhances tumorigenicity in mouse fibroblasts (6). This suggests that drugs that inhibit these enzymes might be able to suppress invasive and metastatic behavior.

Batimastat (also known as BB-94) is a low molecular weight, synthetic inhibitor of MMPs. The structure contains a peptide backbone similar to the cleavage site in collagen, which is bound by the MMP, and a hydroxamate group, which binds the zinc ion in the catalytic site of the MMP, thereby inactivating it.

Several promising studies have been published indicating the antitumor activity of batimastat. Daily i.p. administration of batimastat to nude mice has resulted in reduction of ascites and prolonged survival against human ovarian tumor xenografts (7) and has inhibited metastatic spread in a B16 murine melanoma (8) and a human colon carcinoma model (9). In addition, batimastat inhibited the growth of an orthotopic human colon tumor xenograft (10) and a s.c. hemangiomata (11), indicating a possible antiangiogenic property of this drug.

In the current study, we were interested in studying the site-specific role of MMPs in breast cancer tumor progression.

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3 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; IMEM, Iscove’s MEM; kDa, kilodalton.
In our model, we used the human breast cancer cell line MDA435/LCC6. The MDA-MB-435 cell line, from which the MDA435/LCC6 line was derived, is one of the most invasive and metastatic human breast cancer cell lines ever described. The cell line is highly invasive in in vitro systems and forms aggressive, invasive, metastatic tumors in vivo in the nude mouse (12). It appears to be an adenocarcinoma that has undergone an epithelial-mesenchymal transition (13). The MDA435/LCC6 line was specifically selected in vivo for ascites growth (14). Thus, this line will form either solid tumors or malignant ascites in nude mice, depending on whether the cells are implanted s.c. or i.p. We compared the effect of batimastat on tumor progression for both solid tumor growth and ascites formation with this cell line. We observed that inhibition of MMPs by batimastat in these models produced divergent results on tumor growth. The data suggest that local host factors play a significant role in determining tumor response to this mode of therapy in breast cancer.

MATERIALS AND METHODS

**MDA435/LCC6 Cell Line.** The cell line used in these experiments originated from a malignant ascites resulting from metastasis of a s.c. MDA-MB-435 tumor in nude mice (12, 14). The tumor was subsequently passed as ascites in nude mice by diluting ascites tumor cells with IMEM and injecting $10^6$ cells into a carrier nude mouse. These cells were also passed in vitro in IMEM with 5% FCS. To obtain conditioned media from these cells in vitro, cells were washed with PBS containing BSA twice and then grown in IMEM with HEPES buffer, insulin-transferrin-selenium supplement, fibronectin (1 mg/liter), nonessential amino acids, and glutamine for 48 h.

**Mice.** Female 6–8-week-old NCr nu/nu (athymic) mice were purchased through the National Cancer Institute and housed in an animal facility and maintained according to the regulations set forth by the U.S. Department of Agriculture, the American Association for the Accreditation of Laboratory Animal Care, and the Georgetown University Animal Care and Use Committee. Mice with ascites tumors were weighed every other day, whereas mice with solid tumors were weighed weekly to determine appropriate drug dosing.

**Tumor Implantation.** For the solid tumor model, ascites from a carrier mouse was diluted with IMEM to $3.3 \times 10^6$ cells/ml, and 0.15 ml (i.e., $0.5 \times 10^6$ cells) was injected s.c. between nipples 2 and 3 on each side of the mouse. Tumor volume was calculated by multiplying length $\times$ width $\times$ height $\times 0.5$. For the ascites model, an ascites tumor from a carrier mouse was diluted with IMEM to $6.7 \times 10^6$ cells/ml, and 0.15 ml (i.e., $10^6$ cells) was injected i.p., into nude mice. Tumor growth in the ascites model was monitored by the percentage of total weight gained, and animals were sacrificed when they became moribund. Mortality for the purposes of determining survival was established by the percentage of weight gained. Tumor volumes over a treatment group were averaged, and Student’s $t$ test was used to calculate $P$ values. Animals were sacrificed on day 30 or earlier if tumors became ulcerated.

**Batimastat Treatment.** Batimastat was obtained from British Biotech, Ltd. (Oxford, England). Batimastat was received as either an unformulated drug (for one of the ascites experiments), which was sonicated into suspension with PBS and 0.01% Tween 20 to a concentration of 2.5 mg/ml, or it was received as clinically formulated material (containing methylcellulose, ethanol, polyethylene glycol, and 20 mg/ml batimastat), which was diluted to 5 mg/ml batimastat in 5% dextrose (used for all other experiments). Control solutions of vehicle only were also prepared. Animals were injected with 50 mg/kg (at 5 mg/ml) batimastat i.p. on the days indicated. Day 0 treatments took place immediately after tumor implantation.

**Sample Preparation.** Plasma samples were obtained by cardiac puncture and stored at $-70^\circ$C with 15–20 mm EDTA. Ascites fluid was withdrawn from mice and centrifuged, and the supernatant was stored at $-70^\circ$C. A protein extract from solid tumors was made from excised tumors that were frozen quickly in liquid nitrogen and then stored at $-70^\circ$C. The extract was made by pulverizing tumor in Tris-buffered saline with 1% Triton X-100 and 1 mm CaCl and then concentrated and purified with gelatin-Sepharose beads. The solid tumor protein extract was eluted from the gelatin-Sepharose 4B beads (Pharmacia, Uppsala, Sweden) using the sample buffer described below.

**Zymography.** Samples were prepared as indicated above and analyzed by zymography according to the protocol provided by NOVEX (Novel Experimental Technology, San Diego, CA). This is a modification of the technique described by Heussen and Dowdle (15). Ascites and plasma samples were diluted so that the equivalent of 1 ml was loaded per well. Solid tumor extracts were loaded as collected and not normalized for protein levels. The $2\times$ sample buffer was composed of 0.125 m Tris-HCl (pH 7), 20% glycerol, 4% SDS, and 0.005% bromophenol blue. Samples were electrophoresed on a 10% Tris-glycine acrylamide gel with 0.1% gelatin incorporated as a substrate under nonreducing conditions. The gels were washed with Tris-buffered saline containing 2% Triton X-100, then incubated overnight at 37°C (in an incubation buffer containing 50 mm Tris, 5 mm calcium chloride, 1% Triton X-100, and 150 mm sodium chloride) to allow gelatin degradation. The bands were visualized by fixing and staining the gel with 0.15% Coomassie blue R250 in 20% methanol/10% acetic acid solution and then destaining in the methanol/acetic acid solution without Coomassie blue. Gelatinases A and B (also known as MMP-2 and -9, respectively) were detected in this manner. Human control standards for the gelatinases were made by collecting conditioned media from the human breast ductal carcinoma BT549 cell line stimulated with 10$^{-7}$ m 12-O-tetradecanoylphorbol 13-acetate (Sigma Chemical Co.) for human gelatinase A and the human breast ductal carcinoma cell line HS578t for human gelatinase A. Mouse control standards for mouse gelatinase A and B were prepared from BALB/3T3 cells stimulated with 10$^{-7}$ m 12-O-tetradecanoylphorbol 13-acetate. MMPs were activated where indicated with 1 mm p-aminophenylmercuric acetate (Sigma) for 20 min at 37°C prior to gel loading. In vitro inhibition of the gelatinases was accomplished by adding the indicated amount of batimastat to the incubation buffer during the incubation step to prevent activity of the gelatinases.

**Western Blotting.** Samples were electrophoresed under denaturing conditions on a Tris-glycine 10% acrylamide gel (purchased from NOVEX) and electroblotted onto nitrocellulose membranes. Membranes were incubated with the indicated
monoclonal antibodies against human gelatinases A and B (IM07L and IM10, respectively; Oncogene Science, Cambridge, MA). Horseradish peroxidase-labeled antimouse antibody from sheep (Amersham, Arlington Heights, IL) was used as a secondary antibody and detected with enhanced chemiluminescence (Amersham).

Assessment of in Vitro Cell Growth with Treatment of Batimastat. MDA435/LCC6 cells were plated in vitro in 96-well plates (Costar, Cambridge, MA) at 1500 cells/well. After 24 h, the indicated concentration of drug was added to the wells. After 4 and 6 days, the cells were fixed and stained with 0.5% crystal violet (in a 25% methanol solution) for 20 min and then rinsed with water. After allowing at least 1 day to air dry, the crystal violet in the stained cells was solubilized with 0.1 M sodium citrate in 50% ethanol, and the absorbance was read at 540 nm against a reference wavelength of 405 nm.

Histology. Solid tumors and organs were fixed in 10% formalin for 24 h and then transferred to 70% alcohol for storage. Specimens were paraffin embedded and stained with H&E, periodic acid-Schiff, or Masson's trichrome stain. Ascites fluid was smeared across a slide, allowed to air dry, and stained with H&E.

RESULTS

Effect of Batimastat on Solid Tumor Growth. MDA435/LCC6 human breast cancer cells (0.5 × 10^6) were injected s.c. into each group of 10 athymic nude mice at two sites per mouse. All of the injection sites developed tumors. Mice were injected with 50 mg/kg batimastat or a vehicle-only control solution every day beginning on the same day of tumor implantation through day 28. Tumor volumes were measured at days 17, 26, and 30 after tumor implantation. Animals treated with batimastat had mean tumor volumes of 19.6 (SD, 11.3), 33.05 (SD, 21.3), and 48.6 (SD, 27.6) mm^3 on days 17, 26, and 30, respectively. Control animals had mean tumor volumes of 32.95 (SD, 22.85), 65.95 (SD, 41.6), and 111.3 (SD, 63.1) mm^3, respectively (Fig. 1A). Differences in the mean tumor volumes between treatment groups were significant at days 26 (P < 0.05) and 30 (P < 0.01) using Student's t test. This experiment was repeated twice with nearly identical results.

Considerable variation was seen in the activity of the gelatinases as visualized by zymography (Fig. 1B). However, no distinguishing differences can be seen between the control and treated groups of solid tumor protein extracts. Samples from both groups indicate the presence of inactive gelatinases, with a small amount of active forms. Proteins were identified by comparison of standards from cell lines grown in vitro (see “Materials and Methods”). We noted that although the samples appeared to migrate similarly to the standards from a mouse cell line, the MMP-9 band migrated slightly higher than the standard derived from a human cancer cell line (indicated in Fig. 1B).

Histopathology of solid tumors from both treatment groups showed a cellular mass with evidence of central necrosis and suggested no difference in invasiveness of the treated and control tumors (data not shown). In both groups, the tumor appeared to be invading into the muscular wall of the pleural cavity.

Effect of Batimastat on Ascites Formation. Fifteen athymic nude mice were injected i.p. with 10^6 MDA435/LCC6 human breast cancer cells. Ten of the mice were treated with 50 mg/kg batimastat i.p. every other day from days 5 to 25. The 5 control mice received an equivalent volume of vehicle on the same days as treated mice. Among the control mice, 4 of 5 developed ascites tumors, whereas one remained healthy throughout the experiment. Eight of the 10 treated mice developed ascites tumors within 35 days; 1 lost weight and became cachectic; and 1 developed an ascites tumor after 42 days. As
Site-dependent Effect of Batimastat on Tumor Progression

Fig. 2. A, survival curve for animals with ascites tumors treated with batimastat. Athymic nude mice were injected i.p. with 1 million MDA435/LCC6 human breast cancer cells on day 0. i.p. treatment of 10 mice with 50 mg/kg batimastat and 5 mice with a control solution was begun on day 5 and continued every other day to day 25. B, zymography of ascites fluid from animals treated with vehicle only (left four lanes) and batimastat (right four lanes). The markers indicate where the human gelatinase standards migrated; note that the 92-kDa marker runs slightly ahead of the major mouse gelatinase B band.

shown in Fig. 2A, no significant differences in survival times were seen between treated and control animals.

Ascites fluid collected at the time of death for each animal showed some variation between treated and control animals in gelatinase activity as measured by zymography, with increased levels of latent gelatinase B apparent in most controls and few treated animals (Fig. 2B). Again, proteins were identified by comparing them with known standards from in vitro cell lines. Active forms of gelatinase B are apparent for both treated and control tumors; however, active forms of gelatinase A are not visible.

Although peritoneal fluid accumulation appeared similar in treated and control mice prior to animal death, a dramatic difference was seen in gross tumor cell morphology during animal necropsy. In almost all of the treated mice, the ascites fluid appeared less viscous and less cellular, as evidenced in Fig.

3A, although tumor cell adhesions to peritoneal surfaces, such as intestinal mesentery, diaphragm, and other peritoneal serosal surfaces, were observed, indicating that batimastat did not effect an obvious reduction of overall tumor burden (Fig. 3B). Deposits of batimastat along peritoneal surfaces were grossly evident. This experiment was repeated twice with nearly identical results.

Effect of Batimastat on in Vitro Cell Growth. Batimastat did not significantly inhibit cell growth after 4 days at drug concentrations ranging from 50 nm to 1 µM in MDA435/LCC6 cells grown in vitro. Batimastat at concentrations up to 5000 nm also did not inhibit growth of other tumor cell lines tested (data not shown).

Expression of Gelatinases by MDA435/LCC6 Cells in Vitro. Ascites fluid, including cellular material, was taken from mice implanted with the MDA435/LCC6 i.p. ascites tumors. The cells were rinsed with PBS, and conditioned media were collected from these cells, concentrated, and analyzed by zymography. No detectable gelatinase B activity was found, and very little gelatinase A was noted at the highest concentrations of conditioned media (Fig. 4).

Monoclonal antibodies that recognize human but not mouse gelatinases A and B were used to examine the gelatinolytic activity seen in the ascites fluid and solid tumor extracts by Western analysis (Fig. 5). Samples were run under denaturing conditions and blotted to nitrocellulose. Mouse monoclonal antibodies were used to detect the gelatinases and horseradish peroxidase-like antibodies against mouse IgG. Enhanced chemiluminescence was used to visualize the gelatinases. Gelatinases A and B were detected in the lanes containing the positive controls, conditioned media from human gelatinase-producing cell lines. Under reducing conditions, latent but not activated forms of the MMPs are recognized by the antibody. No bands were detected in the region of gelatinolytic activity seen by zymography, strongly suggesting that the gelatinolytic activity was not the result of human gelatinase A or B. Bands seen at lower molecular weights in the tumor sample lanes represent endogenous IgG detected with the secondary antibody.
Inhibition of Gelatinases by Batimastat \textit{in Vitro}. We next wished to demonstrate that batimastat inhibits gelatinase activity, regardless of its human or murine source. Samples from conditioned media of gelatinase-producing cell lines and tumor protein extracts were run on a zymography gel, and gelatinolytic activity was inhibited \textit{in vitro} by incubating the gels overnight in varying concentrations of batimastat (Fig. 6). Almost all gelatinolytic activity appears to be inhibited in all samples at greater than 5 μM concentrations of batimastat.

**DISCUSSION**

In our solid tumor model, systemic treatment of batimastat (by administering the drug i.p. at a site removed from the tumor) retards the growth of s.c.-implanted tumor cells. The mechanism by which this occurs in our model remains unclear. Because these tumors are not well vascularized, significant differences in the degree of vascularity between control and treated tumors could not be readily determined. It is possible that the differences in tumor growth may be due to an antiangiogenic property of batimastat: small changes in tumor vascular perfusion could lead to larger differences in tumor growth. Despite the smaller tumor sizes in the treated animals, these tumors appeared as invasive as the larger control tumors. Differences in encapsulation of the tumors were not obvious. These findings together suggest that batimastat was not effective in abrogating tumor cell invasion. Although somewhat surprising, our results are quite consistent with another recent study in which a metalloproteinase inhibitor (TIMP-1) was overexpressed in a melanoma cell line. In that study, the authors found that MMP inhibition did not appear to affect extravasation of tumor cells from blood vessels; however, the resultant metastases were growth inhibited by the TIMP-1-overexpressing cells (16). Thus, inhibition of tumor growth by batimastat or TIMP-1 may indicate a role for MMPs in tumor growth not necessarily linked to invasive properties conferred by the MMPs. In addition, a recent batimastat study also used the MDA-MB-435 cell line to study tumor regrowth after primary tumor resection (17). This study also showed tumor growth inhibition with adjuvant batimastat treatment, although the model system appears slightly different. Interestingly, they found that their cell line does produce the gelatinases MMP-2 and -9 \textit{in vitro}. Because our cells do not seem to produce these MMPs, it is notable that we continue to see a growth inhibitory effect by batimastat, indicating its effectiveness in inhibiting stromal MMPs.
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Corresponded to suppression of overall tumor growth and a decrease in ascites fluid accumulation. Cells treated with batimastat appeared encased within stromal tissue. This observation is consistent with the conversion to solid tumor seen in our studies. The lack of activity of batimastat against ascites fluid accumulation seen in our model should not be surprising. Therefore, the lack of activity of batimastat against ascites fluid accumulation is not obvious how batimastat could have a therapeutic impact on ascites fluid accumulation may be the result of a secreted tumor vascular permeability factor increasing influx of a plasma exudate into the peritoneal cavity. Based on these studies, it is not obvious how batimastat could have a therapeutic impact on ascites fluid accumulation. However, the stromal environment within the peritoneal cavity still appears to play a significant role in tumor morphology, as evidenced by the conversion to solid tumor seen in our studies. Both our study and that of Davies et al. (7) are consistent with the ability of metalloproteinases to modulate that environment.

In our study, we used a novel dual model, the same breast cancer cell line growing in two very different environments, which proved useful in illustrating differences in the therapeutic response to batimastat. Pleural effusion is a common manifestation of metastatic cancer of the breast. Although pleural effusion has been difficult to model, growth of breast cancer cells within the peritoneum of the mouse may provide some insight for the study of possible therapeutics. Malignant ascites is a less common manifestation of breast cancer metastasis but is a more common later-stage disease with cancers originating in the abdominal and pelvic cavities. Although human breast cancers have been orthotopically implanted in athymic nude mice, this model gives us the unusual opportunity to look at the same types of cells in two differing sites, representing two distinctly different forms of the same disease.

In our ascites model, treatment with batimastat directly into the peritoneal cavity, at the site of tumor implantation, did not suppress ascites formation, nor did it extend survival. This is in contrast to another i.p. cancer model reported in the literature. Davies et al. (7) found that batimastat inhibited ascites formation in nude mice implanted with ascites-forming ovarian carcinoma xenograft. In that ovarian carcinoma model, tumor cells treated with batimastat appeared encased within stromal tissue when treated with batimastat. This morphological change corresponded to suppression of overall tumor growth and a significantly increased survival time. Interestingly, in almost all of our treated animals (at a dose higher than that used by Davies et al.; Ref. 7), there appeared to be a conversion of the bulk of the tumor from growth as a diffuse suspension in the ascites fluid to growth as adhesive, invasive clumps along peritoneal surfaces (Fig. 3B). When this experiment was repeated with more frequent dosing of batimastat, this effect persisted. Therefore, we conclude that although the activity of this drug was not sufficient to inhibit accumulation of ascites fluid in the model, there was moduation of the tumor morphology in a gross, visible way.

Studies by Nagy et al. (18–21) indicate that ascites fluid accumulation may be the result of a secreted tumor vascular permeability factor increasing influx of a plasma exudate into the peritoneal cavity. Based on these studies, it is not obvious how batimastat could have a therapeutic impact on ascites fluid accumulation. However, the stromal environment within the peritoneal cavity still appears to play a significant role in tumor morphology, as evidenced by the conversion to solid tumor seen in our studies. Both our study and that of Davies et al. (7) are consistent with the ability of metalloproteinases to modulate that environment.

Of the MMP, gelatinases A and B (also known as MMP-2 and 9 and as the 72- and 92-kDa type IV collagenases) have received the most attention for their role in tumor invasion and metastasis. Correlations have been drawn between expression of gelatinases A (22–26) and B (23, 27) with tumor grade and of activation with metastatic potential (28). In addition, the endogenous metalloproteinase inhibitors TIMP-1 and TIMP-2 are believed to be more selective for gelatinases B and A, respectively. Although batimastat inhibits the known MMPs, such as collagenases, stromelysins, and gelatinases, in the nanomolar range (29), we chose to concentrate on gelatinases A and B for our studies as likely significant targets for MMP inhibition.

From the zymography studies, we show that although the MDA435/LCC6 cells do not produce measurable gelatinases A and B in vitro, these MMPs are present in the tumor environment in the in vivo setting. Western blot analysis to detect human gelatinases indicated an absence of detectable human gelatinases A and B in the ascites fluid and in the solid tumor extract. The MMPs expressed in vivo, in the ascites fluid, and in
the tumor extracts appear to be of mouse and not human origin. Mouse gelatinase B migrates at 105 kDa (30, 31), as opposed to human gelatinase B, which migrates at 92 kDa, which is consistent with our findings. Because of these results, we believe that stromal and not tumor MMPs may be the principle targets for batimastat inhibition.

Induction and/or activation of MMPs is believed to be a feature of tumor-stromal interactions in a variety of models (32–36). Tumor cells may induce stromal cells to secrete gelatinases or may themselves be induced to secrete gelatinases by stromal components. In addition, tumor cells may be induced to express gelatinases by contact with the extracellular matrix (37). Our results suggest that tumor induction of stromal gelatinase production may be more important in this tumor model.

The differing effects of batimastat treatment in these two models are extremely interesting. A number of differences in the stromal environment may be responsible for the differing responses to batimastat. For example, differential dependence on angiogenesis between the ascites and solid tumor may explain the difference in effectiveness of batimastat treatment. Because the same cells were used to implant the tumors in these two models, their differing tumor morphology and response to drug treatment may be attributed to differences in the local host environment. More interestingly, the prime candidates to be modulated by this drug, the gelatinases, are from the host (stroma) and not tumor derived. The differences in the drug effects between these two models may reflect the different stromal environments in which these tumors were implanted. The antitumor activity of batimastat is most evident in our solid tumor model, indicating a site-specific action of this drug.

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