Oncolytic Potential of a Novel KGFR Tyrosine Kinase Inhibitor Using a KGFR-selective Breast Cancer Xenograft Model

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Abstract. *Background: Keratinocyte growth factor (KGF)/ KGF receptor (KGFR) signaling produces a rapid increase in the progression of breast cancer. Molecular modeling was used to create a group of KGFR-selective kinase inhibitors (TKI). Compound L-27 is a potent and selective KGFR TKI. The present study examined the oncolytic potential of L-27 using a breast cancer xenograft model. Materials and Methods: An orthotopic xenograft model was developed with KGF-transfected MCF-7 cells to examine the influence of L-27 upon KGFR-mediated tumor progression. Results: L-27 was found to produce a dose-related reduction in the growth and metastasis of mouse xenograft tumors. Furthermore, L-27 treatment did not produce any signs of gross toxicity. Conclusion: L-27 was found to reduce the growth and metastasis of MCF-7 tumor xenografts with elevated expression of KGF. Thus, KGFR TKI may provide a new therapeutic approach for the treatment of breast and other types of cancer.*

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family (also designated FGF7) that is produced in stromal tissue and stimulates DNA synthesis, proliferation and migration of epithelial cells (1-3). It is well-established that target epithelial cells contain high affinity KGF receptors (KGFR) (4, 5). These biological actions of KGF are involved in normal morphogenesis and tissue repair; however, it is known that overexpression of KGF or KGFR is associated with the progression of several types of cancers (3, 6-8). KGF acts at the KGFR and stimulates epithelial cell DNA synthesis, proliferation and migration in breast and other tissue (1, 3, 5). The mammary glands of adult female animals are remarkably sensitive to KGF (9). Systemic administration of KGF to adult male and female rats for 3-5 days was found to produce massive mammary ductal hyperplasia and an increase in the number of mitotic figures (9). Similarly, Kitsberg and Leder observed that female mice with a constitutively up-regulated KGF transgene developed mammary epithelial hyperplasia and eventually all animals developed metastatic mammary carcinomas (10). Consistent with this concept, a high level of KGF expression was observed in human primary breast tumor specimens (11). It has also been reported that KGF is a paracrine growth factor in breast cancer (12). However, highly malignant, metastatic breast cancer tissue expressed relatively small amounts of KGFR (13). We observed that KGF treatment produced a profound stimulation of cell motility and up-regulation of the *KGFR* gene in estrogen receptor (ER)-positive breast cancer cells. This effect did not occur in ER-negative cell lines (14, 15). Taken together, these observations suggest that KGF-mediated stimulation of breast epithelial proliferation and migration may be an important early event in the molecular cascade which leads to cancer progression and metastasis (16).

Previously, we examined a group of 53 KGFR TKIs and L-27 was identified as one of the most potent and selective inhibitors of KGF-mediated breast cancer cell proliferation and extracellular-signal-regulated kinase (ERK) signaling in an *in vitro* model of human breast cancer (17). In addition, it was determined that L-27 reduced the membrane density of KGFR in these breast cancer cells. Thus, the current project examined the potential therapeutic significance of L-27 using a xenograft human breast cancer model specifically designed to demonstrate the effect of KGFR TKI on the progression of KGF-mediated breast cancer.

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Materials and Methods

Cell culture. MCF-7 human breast cancer cells obtained from the Michigan Cancer Foundation (Detroit, MI, USA) were maintained as monolayer cultures in RPMI-1640 media (without phenol red) supplemented with 2 mM L-glutamine, gentamicin (50 pg/ml), penicillin (100 units/ml), streptomycin (100 pg/ml), estradiol (10-11 M) (all from Sigma, St. Louis, MO, USA) and bovine calf serum (5%)(Hyclone, Logan, UT, USA), as previously reported (15).

Nude mouse xenograft model. Female *nu/nu* Balb/C mice at six weeks of age, implanted with the T9 clone of MCF-7 cells, were employed as an *in vivo* model in this study. This animal xenograft methodology was approved by the University of Oklahoma, Health Sciences Center, Institutional Animal Care and Use Committee (protocol # 07-007). The T9 clone has been stably-transfected with β-gal and green fluorescent protein (GFP) reporters and a KGF-producing plasmid (17). The T9 clone has been characterized and shown to express the GFP reporter, as well as a relatively high level of KGF. A separate control clone characterized to express the GFP reporter at the same level as the T9 clone, but without the KGF-producing plasmid, was used as a KGF-negative control. The KGF-transfected T9 clone was shown to be stably transfected and to express and secrete nanomolar quantities of KGF, approximately 500- to 1000-fold greater than the control clone of MCF-7 cells. Furthermore, it was observed that the average tumor size in mice implanted with the T9 clone was fourfold larger than that of mice implanted with the control clone (18). In addition, fluorescent micrographs of liver sections revealed numerous micrometastases in lung and liver sections from mice implanted with KGF-producing T9 cells, while very few micrometastases were found in the liver tissue from mice implanted with control-transfected cells (18, 19).

Approximately 5×10^6 viable T9 or control tumor cells were embedded in 0.2 ml of Matrigel. The gels were implanted subcutaneously, adjacent to mammary fat pads in order to create an orthotopic-type tumor xenograft model. Two days before gel implantation, a 100 g pellet of estradiol was subcutaneously placed on the side opposite tumor cell implantation. Following the implantation of the tumor xenograft, the mice were treated with daily intraperitoneal injections of 4,10, 25 mg/kg of L-27, or vehicle alone, which served as the control. Phosphate-buffered saline was used as the vehicle in this study.

Mice were monitored daily, weighed, and tumors measured by external caliper and by fluorescent imaging. Tumor volumes (mm^3) were calculated using the formula: $(\text{short diameter})^2\times(\text{long diameter})/2$. At the conclusion of the experiment, the mice were killed and dissected for tumor, lung and liver tissue samples. GFP fluorescence of the tumors was confirmed and frozen tissue sections from lung and liver samples were examined for evidence of metastasis as indicated by GFP fluorescence. In order to quantify the relative fluorescence of each sample, Photoshop software was used to crop and convert photographs of GFP fluorescent tumors into black and white jpeg images. ImageJ (software available from nih.gov) was then used to adjust the threshold of these jpeg images to their default parameters and then to quantify the total fluorescent area of each tumor sample.

Statistical analysis. Multiple group-wise comparisons were conducted using ANOVA and Student's *t*-test for pairwise comparisons. Group differences resulting in *p*-values of less than 0.05 were considered to be statistically significant.

Results

Effect of L-27 on early tumor development. In the first xenograft experiment, the effect of L-27 on early tumor development was examined. A timeline of events and graph of average tumor volumes and mice weights is shown in Figure 1. Day 1 of this experiment was considered the day on which estrogen pellets were implanted into the mice. This was followed four days later with implantation of the transfected MCF-7 cells on day 5. Forty eight hours later, mice were treated with daily injections of either 4,10 or 25 mg/kg of L-27 or vehicle control for seven consecutive days. Beginning on day 13, mice were weighed and tumor volumes measured. On day 41, the final tumor volumes and animal weights were measured and the mice were sacrificed. Over the course of the experiment, tumor volumes decreased significantly in the L-27-treated groups as compared to the control group, whereas animal weights across the four treatment groups were approximately equivalent.

External photographs of the tumor xenografts were taken using fluorescent imaging during the course of the experiment. Mice were photographed on days 14, 27, and 40 corresponding to the time line shown in Figure 1. Relative tumor volumes from a single representative mouse from the control group and from the 25 mg/kg L-27-treated group are shown in Figure 2. The presence and approximate sizes of the tumor xenografts were confirmed by the GFP reporter. Overall, tumor fluorescence appeared to be greater in control mice compared to mice from the treatment groups on days 27 and 40, as represented by the mice in Figure 2.

Effect of L-27 on established tumors. A second xenograft study of the effects of L-27 on more established tumors was conducted. A timeline of events and graph of average tumor volumes and mice weights is shown in Figure 3. Estrogen pellets and transfected MCF-7 cells were implanted as described in the first xenograft experiment. This was followed by 24 days of tumor growth in which the mice were not treated. Following this period of tumor growth, the mice were weighed and randomly divided into four groups (containing five mice each) so that the average animal weight of each group was approximately equal. These mice were then treated for 21 consecutive days with daily injections of L-27 at a dose of either 4 mg/kg, 10 mg/kg, 25 mg/kg, or vehicle in the control group. Day 1 of this experiment was the day in which daily injections of L-27 or vehicle control were initiated.

During this time the mice were weighed and tumor volumes measured. On day 21 the final tumor volumes and animal weights were measured and the mice were sacrificed. Over the course of the experiment, tumor volumes decreased significantly in the treated groups as compared to the controls, whereas animal weights across the four groups were not significantly different.

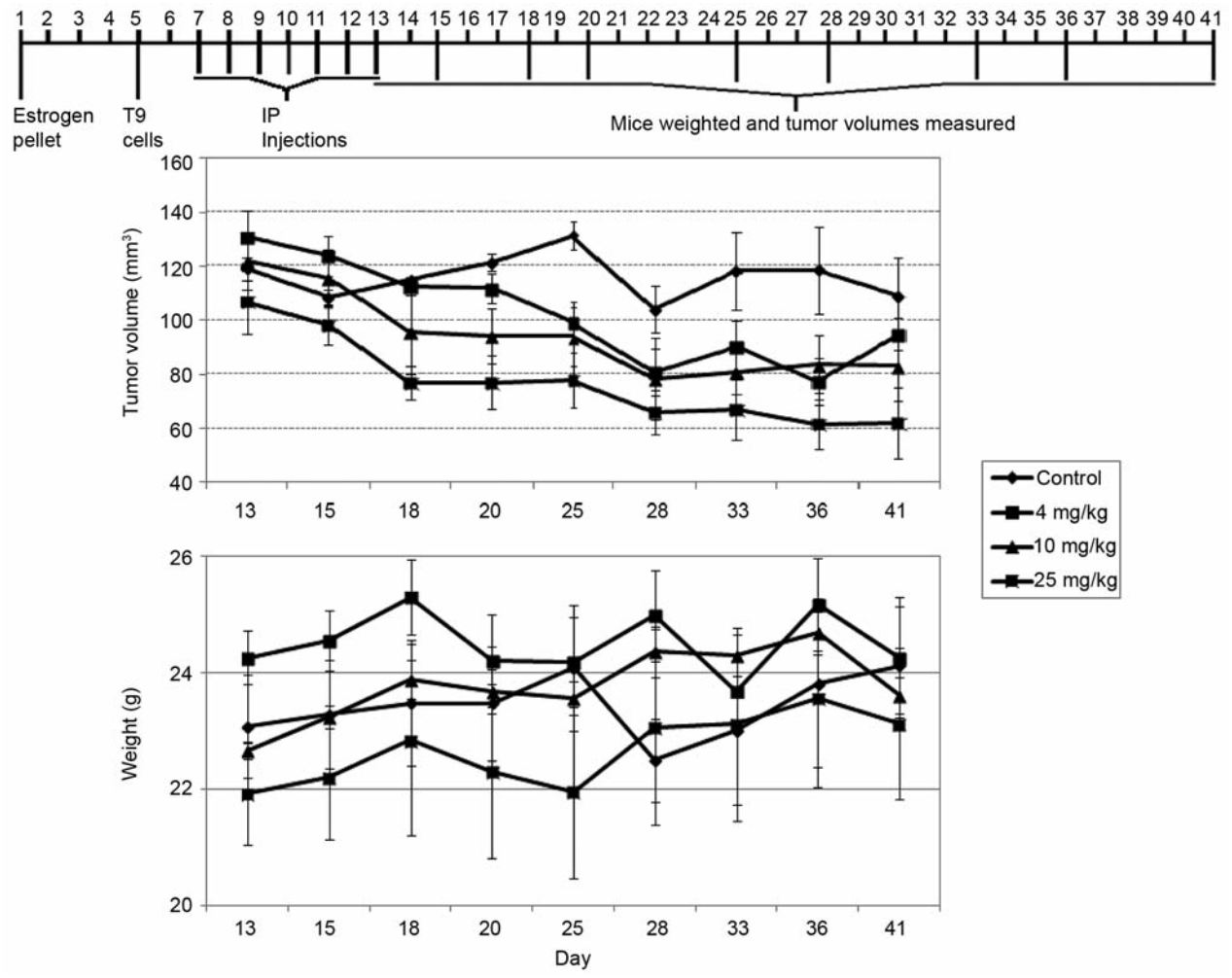

Figure 1. *L-27 treatment during early tumor development. Each point represents the mean±SEM of 5 observations.*

Tissue samples were collected on day 21, at the end of the 3-week treatment period. Tissue fluorescence in lung and liver biopsies from control mice was observed to be much greater than that in samples from mice treated with L-27, as shown in Figure 4. This response to L-27 was observed to be dose-dependent. These results confirm that there was a dose-dependent reduction in the fluorescence of lung and liver sections from L-27-treated mice, compared to tissue from control animals.

Discussion

Up-regulation of KGF and KGFR expression has been observed in human primary breast tumor specimens (11, 14). In addition, there exists evidence that KGF acts as a paracrine growth factor in breast cancer (12). Furthermore, KGF and KGFR have been reported to enhance the progression of breast cancer by inhibiting apoptosis (20).

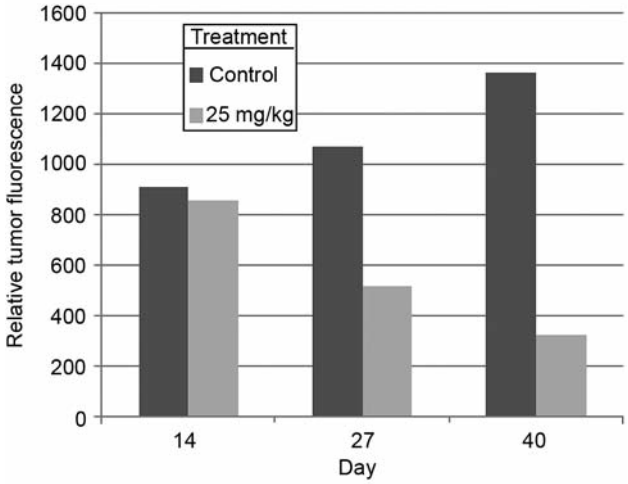

Figure 2. *Fluorescence of green fluorescent protein in tumor xenografts. Each bar represents one observation representative of each group.*

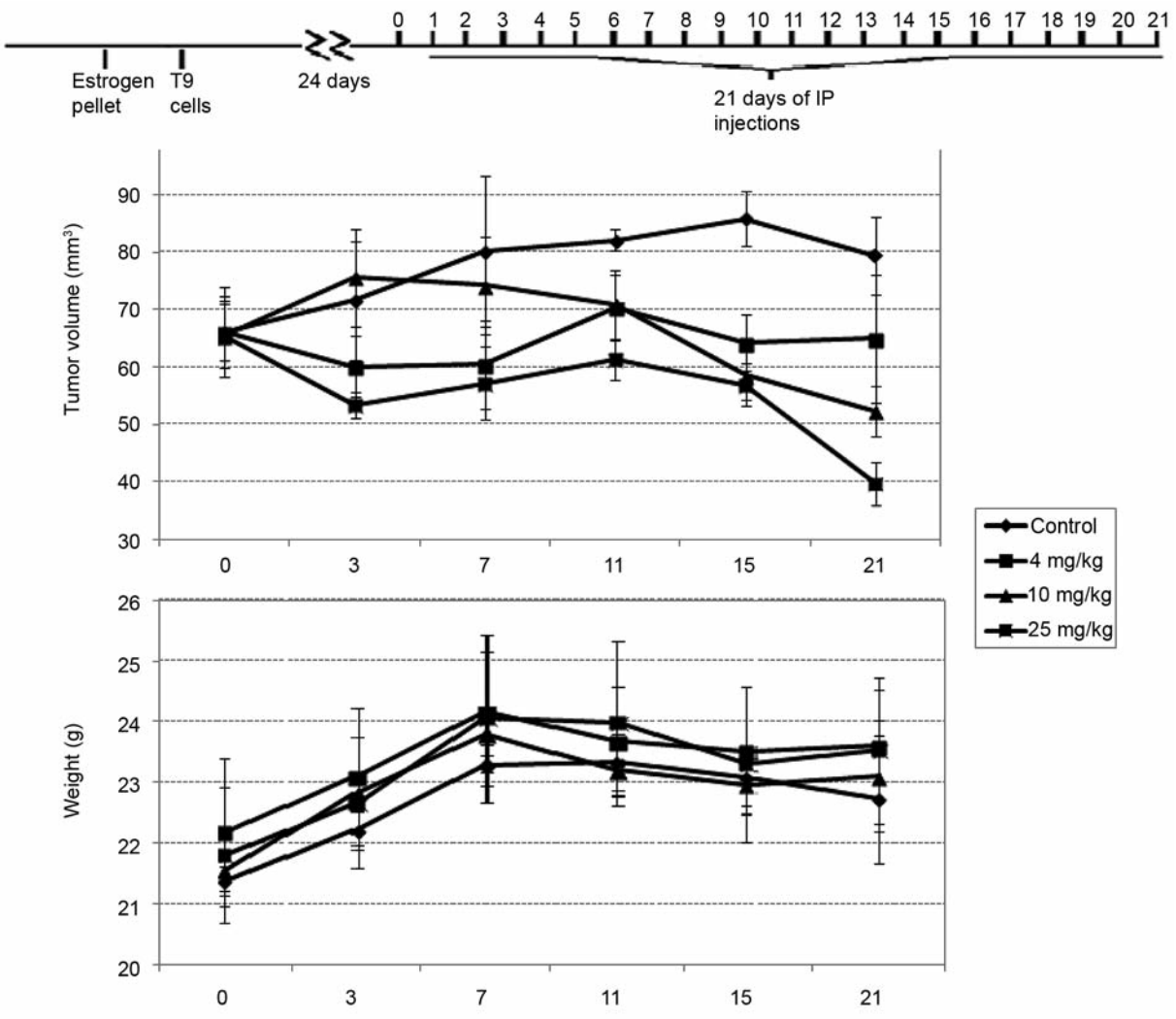

Figure 3. *L-27 treatment of established tumors. Each point represents the mean±SEM of 5 observations.*

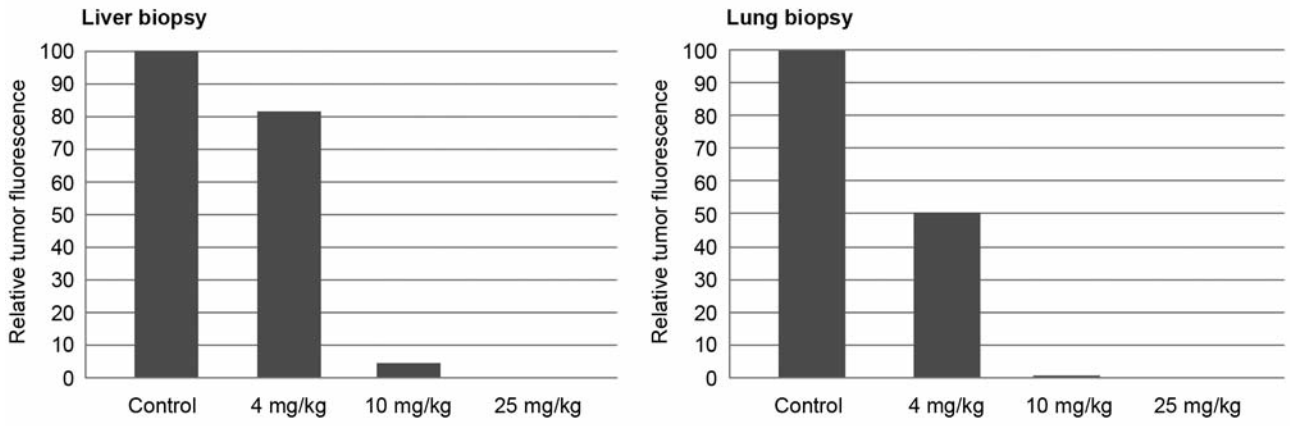

Figure 4. *Fluorescence of green fluorescent protein in lung and liver tissue. Each bar represents one observation representative of each group.*

KGF treatment of ER-positive breast cancer cells *in vitro* produced a rapid increase in tumor cell proliferation and motility and an increased metastatic potential (15, 18). Furthermore, this KGF effect appears to be mediated primarily by activation of KGFR *via* the ERK1/2 signal transduction pathway (14). Thus, the enhancement of KGF/KGFR signaling may be an early event in the progression of breast cancer metastasis (21). It is possible that an increased release of KGF from breast stromal tissue or an up-regulation of KGFR receptor signaling in developing breast cancer tissue may represent an early enabling step in the initiation of metastatic progression (20). Thus, therapeutic approaches, such as selective inhibition of KGFR-mediated activity, may effectively inhibit the growth and early progression of breast cancer to a more malignant and metastatic phenotype, with fewer adverse side-effects than current chemotherapy (22, 23).

Since we previously reported that L-27 is an effective and relatively selective KGFR inhibitor, the present study was designed to determine the efficacy of this compound in reducing or altering cancer growth and progression *in vivo* using a mouse xenograft model of human breast cancer.

Throughout this study, a dose-dependent effect of L-27 treatment on the reduction of tumor volumes was observed. Accordingly, L-27 inhibited both early tumor development and the development and progression of established tumors in a dose-dependent manner. The presence of transfected MCF-7 cells within the tumor xenografts was confirmed using fluorescent imaging of a GFP reporter. Since the average weight of the L-27-treated mice was not different than the control mice at the end of these experiments and no evidence of internal organ toxicity was observed, it appears that L-27 treatment did not produce any gross toxicity in this study. Furthermore, the dose-dependent reduction of GFP in lung and liver samples from L-27-treated animals suggests that this KGFR TKI inhibits the metastatic progression of these human breast cancer cells in this xenograft model. Since wild-type MCF-7 cells are known to be poorly metastatic in mouse xenografts, these results indicate that elevated levels of KGF in the tumor microenvironment of the T9 cells enhanced cancer cell proliferation, tumor growth and the development of numerous micrometastases. The results of this study indicate that this xenograft model, utilizing KGF-expressing cancer cells, is an effective model to provide an accurate assessment of the ability of KGFR TKI to inhibit KGF-mediated metastatic progression.

Furthermore, mutational activation and amplification of receptor tyrosine kinase activity and related signal transduction pathways are known to be involved in the development and metastatic progression of many types of cancers (22, 24). There is evidence that KGF/KGFR signaling is involved in the progression of other types of cancers, such as cervical (25), colorectal (6, 26, 27), ovarian (28), lung (7), stomach (29), endometrial (30-32), and pancreatic (33, 34).

In conclusion, the results of the present study demonstrate the effectiveness of L-27 in reducing the growth and progression of breast cancer in this xenograft model. Thus, these results suggest that that development of KGFR TKI could lead to a new class of molecularly-targeted therapeutic agents used to treat or to prevent cancer growth and metastatic progression in patients with breast cancer or to prevent the occurrence of breast cancer in high-risk individuals. Furthermore, these novel KGFR TKI compounds hold the potential to be useful for the treatment of many other types of cancer in which KGFR signaling has been implicated.

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