Influence of Novel KGFR Tyrosine Kinase Inhibitors on KGF-mediated Proliferation of Breast Cancer

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Abstract. *Background: Keratinocyte growth factor (KGF) acts at the KGF receptor (KGFR) to produce a rapid stimulation of breast cancer cell proliferation and motility which is mediated via the Erk signaling pathway. Enhancement of KGF/KGFR signal transduction may be an early step in the metastatic progression of breast cancer. Receptor modeling of KGFR was used to identify selective KGFR tyrosine kinase (TK) inhibitor molecules that have the potential to bind selectively to the KGFR. The present study evaluated the biological activity of 57 of these KGFR TK inhibitor compounds on breast cancer cells. Materials and Methods: These compounds were tested for their ability to inhibit KGF-mediated breast cancer cell proliferation in MCF-7 breast cancer cells. Furthermore, the effects of the most effective proliferation inhibitors were examined on Erk signaling and on the relative density of cell membrane KGFR. Results: It was observed that 27 of the 57 compounds tested produced a 20% or greater reduction in KGF-mediated proliferation; while five compounds produced greater than 50% inhibition. In addition, the most potent inhibitors also reduced Erk signaling and cell membrane density of the KGFR. Conclusion: The compounds examined appear to be selective KGFR inhibitors which inhibit KGF-mediated activity and reduce the expression of KGFR on cancer cells. These results may lead to the development of a novel class of anticancer agents for the prevention of metastatic cancer progression.*

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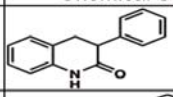
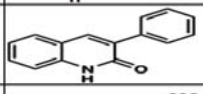
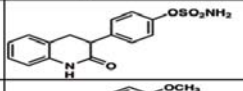
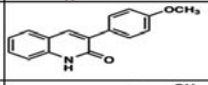
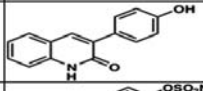
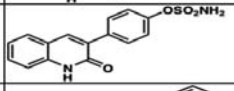
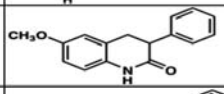
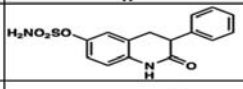
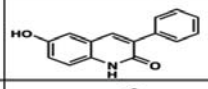
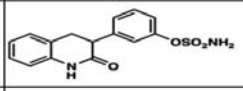
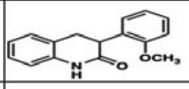
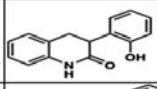
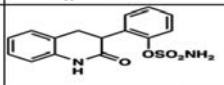
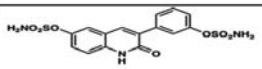
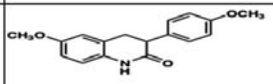
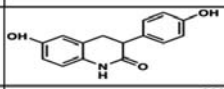
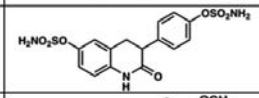
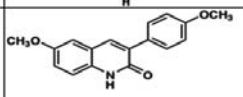
Key Words: Cell proliferation, Erk signaling, keratinocyte growth factor, KGFR tyrosine kinase inhibitor, breast cancer, metastasis prevention.

Keratinocyte Growth Factor (KGF, also designated FGF-7), a member of the fibroblast growth factor family, was originally isolated from human embryonic lung fibroblasts (1). KGF is produced by stromal cells (2) and acts at the KGF receptor (KGFR) found on epithelial cells. KGFR (also known as FGFR-2IIIb) is a splice variant of FGFR-2 encoded by the FGFR-2 gene (3). Thus, KGFR is a member of the fibroblast growth factor receptor (FGFR) family which are membrane-spanning tyrosine kinase receptors consisting of four known peptides whose sequences are highly conserved (4). KGF acts at the KGFR and stimulates epithelial cell DNA synthesis, proliferation and migration in breast and other tissues (2, 5, 6). Accordingly, it has been observed that elevated levels of KGF in female rodent species induced mammary epithelial hyperplasia and the eventual development of metastatic mammary carcinomas (7, 8).

It has previously been shown that KGF treatment up-regulates KGFR gene expression in MCF-7 breast cancer cells (9), and induces rapid and direct motility enhancement in MCF-7 and other estrogen receptor positive breast cancer cell lines (10). In addition, it has been reported that KGF/KGFR-induced proliferation and motility is mediated *via* the Erk1,2 signaling pathway in MCF-7 human breast cancer cells (11).

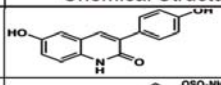
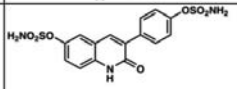
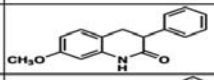
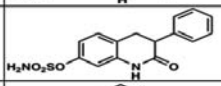
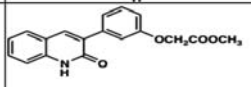
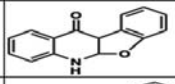
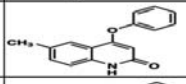
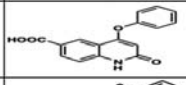
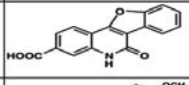
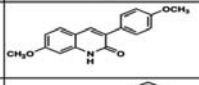
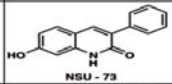
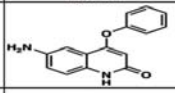
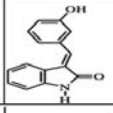
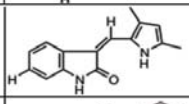
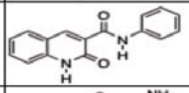
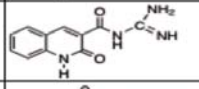
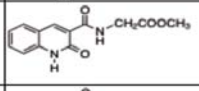
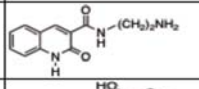
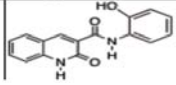
These results suggest that KGF-mediated stimulation of breast epithelial cell proliferation and migration may be an important early event in the molecular cascade, which leads to breast cancer progression and metastasis (12). Thus, the inhibition of KGF/KGFR signaling may be an important therapeutic target to selectively retard the metastatic progression of breast cancer with few, if any, adverse side effects. The objective of the present study was to evaluate the biological activity of a novel group of KGFR TK inhibitors that have the potential to selectively reduce KGF-mediated cancer progression.

Table I. *Effect of KGFR TK inhibitors on KGF-mediated proliferation. Each % reduction value represents the mean of the 3-5 observations.*

KGFR TK Inhibitor	Molecular Weight	% Reduction in KGF-Mediated Proliferation	*p*-Value	Chemical Structure
NSU-1	223	23.13	0.02690	
NSU-2	221	7.58	0.33141	
NSU-5	318	11.48	0.05247	
NSU-6	251	23.21	0.04854	
NSU-7	237	49.05	0.00041	
NSU-8	316	25.4	0.00308	
NSU-9	253	44.77	0.00224	
NSU-11	318	6.11	0.01198	
NSU-13	237	23.23	0.35975	
NSU-17	318	35.93	0.00805	
NSU-21	253	22.95	0.00859	
NSU-22	239	5.84	0.03702	
NSU-23	318	30.73	0.23864	
NSU-32	411	13.5	0.45981	
NSU-33	283	13.25	0.00598	
NSU-34	255	22.1	0.01914	
NSU-35	413	26.61	0.01620	
NSU-36	281	27.72	0.00477	

continued

Table I. *continued*

KGFR TK Inhibitor	Molecular Weight	% Reduction in KGF-Mediated Proliferation	*p*-Value	Chemical Structure
NSU-37	253	29.64	0.06970	
NSU-38	411	5.6	0.0920	
NSU-39	253	31.8	0.04195	
NSU-41	316	38.4	0.01470	
NSU-43	309	3.44	0.15273	
NSU-49	235	83.53	0.00647	
NSU-54	251	30.82	0.04312	
NSU-57	281	45.04	0.00559	
NSU-61	279	26.9	0.00126	
NSU-71	281	3.6	0.0101	
NSU-73	237	31.15	0.00028	 NSU - 73
NSU-80	252	54.72	0.00197	
NSU-118	225	61.23	0.00098	
NSU-129	238	47.52	0.23442	
L-14	264	9.65	0.00896	
L-21	230.2	37.7	0.26520	
L-22	260.2	61.6	0.00066	
L-27	231.3	78.62	0.000009	
L-31	280.1	37.45	0.13937	

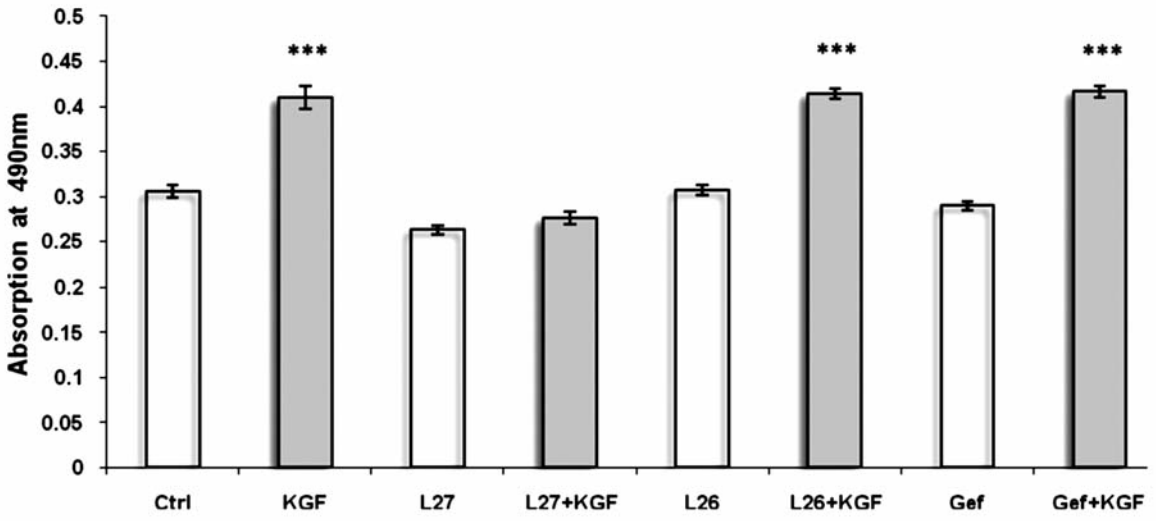


Figure 1. *Effect of KGFR TK inhibitors (20 μM) on KGF-mediated proliferation of MCF-7 at 48 hrs. The bars represent the mean of 3-5 observations ± SEM. Statistical significance; ***p<0.005.*

Materials and Methods

Development of small-molecule ATP-competitive inhibitors as selective KGFR TK inhibitors. A homology model of the KGFR tyrosine kinase domain using 2FGI, the crystal structure of FGFR-1, was previously constructed as a template (16). The protein structure of FGFR-1 has 86% homology with the KGFR tyrosine kinase domain and has been used to guide the design of novel ATP site-directed ligands (13-15). *In silico* site-directed mutagenesis was used to generate a model of the KGFR TK domain as previously described (16).

Using this model, a series of 57 potential inhibitors of KGFR was identified. The series contains compounds with indolinone and quinolinone structural core and their conformationally restricted analogues. Synthesis of these compounds was previously described (16).

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

Cell proliferation assay. One day before treatment, approximately 1000 cells per well were seeded in 96 well plates and allowed to attach overnight. The treatment vehicle contained 0.5% DMSO. Treatment groups consisted of 3-5 wells each. The cells were treated for 48 h with either human recombinant KGF (R&D Systems, Minneapolis, NM, USA) at 50 ng/ml, KGFR TKI at 20 μM or a combination of KGF and KGFR TK at the same concentrations or vehicle alone in the control group. At the end of the 48 h treatment period, the viable cell number was determined using an MTS assay according to the manufacturer's protocol (Promega, Madison, WI, USA).

Erk signaling assay. Two days before treatment, approximately 3000 cells per well were seeded in 96 well plates and allowed to attach overnight. Treatment groups consisted of 3-5 wells each. The cells are treated for 10 min with either KGF at 500 ng/ml, KGFR TK inhibitor at 100 μM or a combination of KGF and KGFR TK inhibitor at the same concentrations or vehicle in the control group. At the end of the treatment period the relative Erk 1/2 phosphorylation was measured using a cell based ELISA assay according to the manufacturer's protocol (Ray Biotech, Norcross, GA, USA).

Immunocytochemistry. Cells were placed on glass slides and air dried for 24 h at room temperature and prepared for immuocytochemistry as previously described (17). The cells were stained for KGFR using the KGFR Bek rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The slides were counterstained with hematoxylin (Vector Laboratories, Burlingmane, CA, USA), cleared with xylene, and coverslipped with Acrymount (StatLab, Lewisville, TX, USA). Cells were processed in the same manner, except without KGFR primary antibody, were included to exclude negative immunoreactivity.

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

Results

Effects of KGFR TK inhibitors on KGF-mediated cell proliferation. It was observed that 27 of the 57 KGFR TK inhibitors tested produced greater than 20% inhibition of KGF-mediated proliferation, while five compounds (NSU-49, NSU-80, NSU-118, L-22 and L-27) produced greater

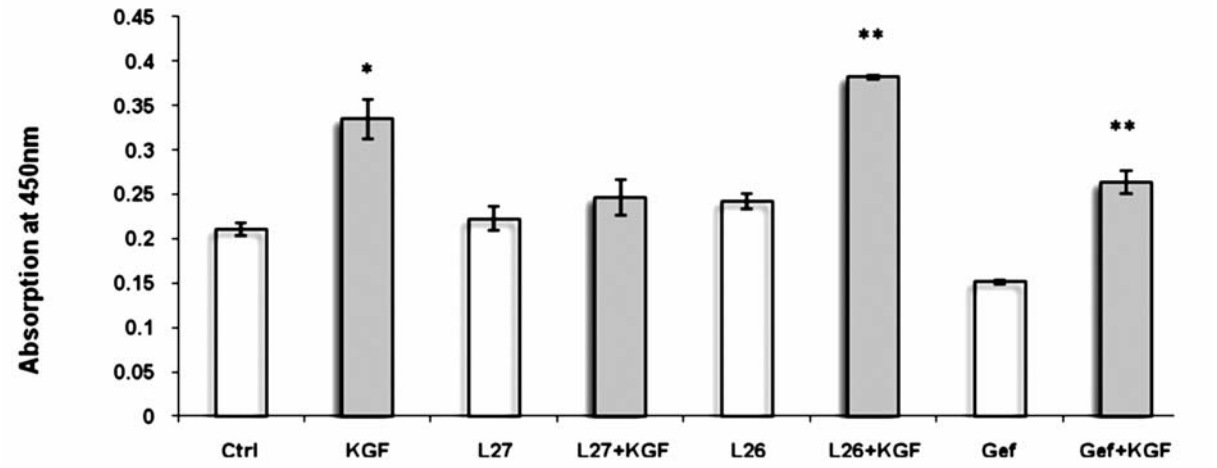


Figure 2. *Effect of KGFR TK inhibitors (100 μM) on KGF-mediated Erk phosphorylation in MCF-7 at 10 min. The bars represent the mean of 3-5 observations ± SEM. Statistical significance; *p<0.05, **p<0.01.*

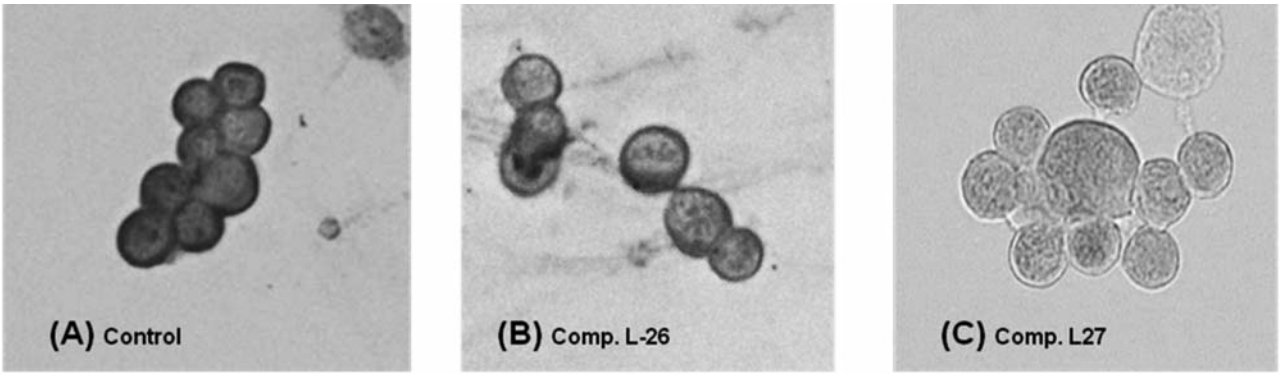


Figure 3. *Immuno-localization of KGFR in MCF-7 cells. In these photomicrographs (×200) MCF-7 cells were treated with either (A) vehicle or (B) compound L-26 (60 μM, negative control) or (C) L27 (60 μM) for 24 h before processing by immuno-cytochemistry. Increased density represents KGFR immuno-localization.*

than 50% inhibition ($p<0.05$) in the breast cancer cells (see Table I). The effects of an active inhibitor (L-27), inactive compound (L-26) and EGFR selective inhibitor (gefitnib) are presented in Figure 1. One of the KGFR TK inhibitors (NSU-129) which inhibited KGF-mediated proliferation (see Table I), produced a 31% increase ($p<0.05$) in proliferation when administered in the absence of KGF (data not shown).

Effects of KGFR TK inhibitors on KGF-mediated Erk signaling. The influence of the five compounds found to be the most potent in reducing KGF-mediated proliferation were examined for their effects on KGF-mediated phosphorylation or activation of Erk. The results indicated that three of the active KGFR TK inhibitors (NSU-80, L-22 and L-27) reduced ($p<0.05$) KGF-mediated phosphorylation, while the inactive KGFR TK inhibitors, those that did not alter KGF-mediated proliferation, did not alter KGF-mediated phosphorylation. The results of an experiment with the active KGFR TK inhibitor (L-27), inactive KGFR TK inhibitor (L-26) and EGFR selective TK inhibitor (gefitnib) are illustrated in Figure 2.

Effects of KGFR TK inhibitors on KGFR immuno-localization. Compounds which were found to be the most potent KGF inhibitors in the cell proliferation assays (NSU-49, NSU-80, NSU-118, L-22 and L-27) produced a marked reduction in KGFR density on the MCF-7 cells as determined by immunocytochemistry. Figure 3 illustrates the effects of L-27 on KGFR receptor density as compared to inactive compound L-26 and a vehicle treated control.

Discussion

Up-regulation of KGF and KGFR expression has been observed in human primary breast tumor specimens (9, 18). Furthermore, there is evidence that KGF acts as a paracrine growth factor in breast cancer (19). Moreover, KGF and KGFR have been reported to enhance the progression of breast cancer by inhibiting normal apoptosis (20).

KGF treatment of ER-positive breast cancer cells *in vitro* has been observed to produce a rapid increase in the proliferation and motility and an increased metastatic potential (10, 21). Furthermore, this KGF-mediated effect appears to be mediated primarily by activation of KGFR *via* the Erk1,2 signal transduction pathway (11). Thus, the enhancement of KGF/KGFR signaling may be an early event in breast cancer metastatic progression (22). Accordingly, specific inhibition of KGF-mediated receptor signaling at the KGFR TK receptor is expected to reduce or eliminate KGF-associated effects on breast cancer motility and metastatic progression.

The present study was designed to examine the effectiveness of a novel group of KGFR TK inhibitors and to identify the most potent and selective inhibitors of KGF-mediated breast cancer cell progression. The results of the biological testing conducted in this study revealed that approximately half of these compounds produced more that 20% inhibition of KGF activity and that five compounds produced greater than 50% inhibition. These biological results compared favorably with the estimated free binding energy of these molecules obtained through molecular screening (16). Furthermore, the reduction of KGFR density on the surface of the cancer cells suggests that interaction of the inhibitor compounds at the ATP binding site of KGFR produces a down-regulation of KGFR expression in the breast cancer cell. This agrees with an earlier observation that KGF treatment enhanced KGFR expression using a cDNA expression array (23). Further analysis of structure-activity relationships, based on these biological results, should permit the creation of a more accurate homology model and result in the identification of even more effective KGFR TK inhibitors.

It is possible that an increased release of KGF from breast stromal tissue, an up-regulation of KGFR or 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.

In conclusion, these results demonstrate that modeling of the KGFR is capable of creating highly effective and selective KGFR TK inhibitors. Furthermore, these compounds may have the potential to be used therapeutically for the reduction or prevention of breast cancer metastatic progression.

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