Abstract

Damage to the gastrointestinal mucosa is a common dose-limiting toxicity of several anticancer therapies. Until recently, adequate control of oral mucositis was considered a significant unmet medical need, with most available treatments providing only palliative benefits without protecting the gastrointestinal epithelium from the damaging effects of cancer therapy. In 2005, palifermin [recombinant human keratinocyte growth factor (KGF)] was approved to decrease the incidence and duration of severe oral mucositis in patients with hematologic malignancies receiving myelotoxic therapy requiring hematopoietic stem cell support. Current trials are investigating the use of palifermin in solid tumor settings. The objective of this study was to determine whether combining palifermin with different chemotherapeutic or biological agents affected the antitumor activity of these agents in human head and neck (FaDu) and colorectal (HT29) carcinoma xenograft models. Nude CD1 mice were injected with \( 1 \times 10^7 \) of either FaDu or HT29 cells, which express both KGF and epithelial growth factor receptors. Animals were treated with palifermin in various combinations with chemotherapeutic (5-fluorouracil and cisplatin) and/or biological (bevacizumab, cetuximab, and panitumumab) agents. Palifermin alone had no effect on either FaDu or HT29 tumor growth. Palifermin did not affect the therapeutic efficacy of 5-fluorouracil, cetuximab, bevacizumab, or panitumumab in any of the two- or three-way drug combinations tested in either model. The results of this study showed that palifermin did not promote the growth of two carcinoma cell lines that express functional KGF receptors and did not protect these tumor cells from the antitumor effects of several chemotherapeutic and biological agents.

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Introduction

The clinical utility of many anticancer agents is limited by their toxic effects on normal tissues, particularly epithelial tissues. The high proliferative rate of the gastrointestinal epithelium makes it particularly susceptible to damage by chemotherapy or radiotherapy (1), resulting in a pathologic condition known as mucositis. The direct cytotoxic effects of therapy can set in motion a cascade of pathologic events within the submucosal and epithelial layers that leads to progressive cell damage and death, mucosal atrophy, thinning of the epithelium, ulceration, and a breakdown of the mucosal barrier function (2-4). Mucositis can be extremely painful, puts patients at risk of potentially life-threatening infections, increases overall health care use, and has been associated with increased overall mortality in cancer patients (4-9). It is considered one of the most common dose-limiting toxicities of some anticancer therapies and can force dose reductions or interruptions in cancer treatment that may contribute to suboptimal clinical outcomes (6, 10-14).

The incidence of mucositis ranges from 5% to 40% in patients receiving chemotherapy for solid tumors such as colorectal cancer (15-17) to near 100% in patients receiving combined radiation and chemotherapy for head and neck cancer (18, 19). The chemotherapeutic agents 5-fluorouracil (5-FU) and cisplatin are associated with particularly high rates of mucositis (11, 20). Until very recently, most of the treatments for mucositis provided only palliative benefits and did little if anything to interfere with the damaging effects of cancer treatments on the gastrointestinal epithelium (21).

Keratinocyte growth factor (KGF) has been shown to stimulate proliferation, migration, differentiation, and survival of epithelial cells (22). It is a member of the fibroblast growth factor family, and KGF (FGFR2IIIb) is predominantly expressed by epithelial tissues (23). Numerous in vitro and in vivo studies have shown that KGF can have cytoprotective and regenerative effects on epithelial tissues exposed to a variety of insults, such as radiation (24, 25), hypoxia (26), and chemotherapy (25, 27, 28). In 2005, a truncated form of recombinant human KGF (palifermin, Kepivance, Amgen, Inc.) was approved to decrease the incidence and severity of oral mucositis in stem cell transplant patients suffering from hematologic malignancies (29), but the safety and efficacy of palifermin in patients being treated for nonhematologic malignancies has not been established.
The use of any KGF-based therapy in patients with solid tumors must be considered with caution as many solid tumors are epithelial in origin and have the potential to express KGFRs (30). Several nonclinical studies, however, suggest that the KGF/kgFR axis does not seem to be a critical pathway promoting tumorigenesis or tumor growth (30-36). In addition, preliminary long-term follow-up data from a clinical study of palifermin in the treatment of oral mucositis in patients suffering from metastatic colorectal cancer found no evidence that palifermin had any effect on the cancer outcomes in patients followed for a median of 14.5 months (27). Nonetheless, a more thorough evaluation of the potential of palifermin to stimulate solid tumor growth or protect tumors from the cytotoxic effects of chemotherapy or targeted biological agents is needed.

The purpose of the present study was to evaluate the effects of palifermin on the antitumor activity of various chemotherapeutic and biological agents in human head and neck (FaDu) and colorectal (HT29) carcinoma xenograft models expressing functional KGFRs and epithelial growth factor receptor (EGFR). The experimental design took into account current clinical practice in the treatment of head and neck and colorectal cancers, evaluating several cytotoxic and antibody-based therapies currently used to treat these cancer types. These included the cytotoxic agents cisplatin and 5-FU, among the most common treatments for head and neck cancer (37, 38) and colorectal cancer (39, 40), respectively. Three new antibody-based therapies were also evaluated and they included bevacizumab, a recombinant humanized monoclonal IgG1 antibody to human vascular endothelial growth factor (Avastin, Genentech), cetuximab (recombinant human/mouse chimeric antibody to human epidermal growth factor receptor; Erbitux, ImClone Systems, Inc.), and panitumumab (recombinant human IgG2κ monoclonal antibody to human EGFR; Vectibix, Amgen). These are all used to treat colorectal cancer (41-48); cetuximab is also used to treat head and neck cancer (38). Moreover, some of these antibody-based therapies are indicated for use in combination with cytotoxic agents such as cisplatin and 5-FU (49-51). The present study was designed to determine whether palifermin had any effect on the antitumor activities of these agents when administered in various combinations. The direct effects of palifermin on tumor growth and gastrointestinal epithelia were also evaluated.

Results

**KGFR and EGFR Expression in Human Carcinoma Cell Lines**

In the process of identifying the best candidates for the xenograft models, we first selected cell lines that were deemed most relevant to ongoing and proposed clinical investigations with palifermin in patients being treated for solid tumors (colorectal and head and neck cancers). From among the appropriate candidates, cell lines were selected that expressed both the KGFR and EGFR. KGFR transcripts were expressed in 15 of 22 human carcinoma cell lines tested and EGFR transcripts were present in 19 of these same cell lines. The FaDu (hypopharyngeal carcinoma) and HT29 (colon carcinoma) cell lines expressed both KGFR and EGFR transcripts (Fig. 1A) and were selected for further evaluation in the tumor xenograft models. These tumor lines showed a good response to the therapeutic agents that were relevant to the clinical settings. Any candidate model had to be responsive to the five antitumor therapeutic agents selected (5-FU, cisplatin, bevacizumab, cetuximab, and panitumumab) as single agents, and this significantly limited our choices.

The expression of KGFR and EGFR transcripts by FaDu and HT29 cell lines was maintained in vivo in both xenograft models (Fig. 1B and C). In vitro, both FaDu and HT29 cell lines expressed functional KGFR and EGFR, which could be stimulated by the addition of KGF and EGF. Activation of both receptors by their respective ligands resulted in significant phosphorylation of protein kinase B (Akt) and extracellular signal-regulated kinase (Erk; Fig. 2). Although these experiments were run under serum-free conditions, which are unlikely to be of physiologic relevance, they were designed in this way to enhance the sensitivity of this assay. This was deliberately done in an attempt to confirm protein expression of both receptors and provide the greatest opportunity to detect an intracellular signal.

**Effects of Palifermin in Combination with Cisplatin and Bevacizumab on the Growth of FaDu Human Head and Neck Carcinoma Xenografts**

Xenografts of FaDu carcinoma cells were implanted subcutaneously in nude mice and allowed to grow for 11 days before the start of treatment. Palifermin (5 mg/kg) was administered for 3 days before and after cisplatin dosing. The 5 mg/kg dose is efficacious in protecting mice from chemotherapy and radiation-induced gastrointestinal injury (25, 52) and the 3-plus-3 dosing regimen was chosen to mimic that used in stem cell transplant patients (53-55). Bevacizumab was dosed twice a week throughout the duration of the experiment based on our previous experience with this dosing regimen in this xenograft model (Fig. 3A). Tumor growth was significantly less in animals treated with either cisplatin, bevacizumab, or the combination of cisplatin and bevacizumab than in animals treated with vehicle (P < 0.0001). The addition of palifermin had no effect on the antitumor efficacy of any of these treatment regimens (P = 0.9803; Fig. 3A).

In animals treated with palifermin alone, the FaDu tumor growth was similar to that seen in animals treated with vehicle (P = 0.9905), indicating that palifermin did not promote tumor growth in this KGFR-expressing carcinoma model (Fig. 3A).

In addition, the effect of palifermin on esophageal epithelial growth was evaluated histologically in each series of experiments with both xenograft models to confirm the biological activity of palifermin under these experimental conditions. In all experimental series, the esophageal epithelium was substantially thicker in mice treated with palifermin compared with those treated with vehicle alone. A representative histologic panel from FaDu tumor-bearing animals treated with vehicle or palifermin is shown in Fig. 4. The data are not shown for the other experiments due to space limitations.

**Effects of Palifermin in Combination with Cisplatin and the Anti-EGFR Antibody Cetuximab on the Growth of FaDu Human Head and Neck Carcinoma Xenografts**

The FaDu xenograft model was also used to study the effect of palifermin on the antitumor activity of the anti-EGFR
FIGURE 1. A. Expression of KGFR and EGFR transcripts in vitro. B. In vivo expression of KGFR in FaDu and HT29 xenograft models. C. In vivo expression of EGFR in FaDu and HT29 xenograft models. Expression in vivo was evaluated only for the FaDu and HT29 tumor xenografts. Three representative animals from each xenograft are shown and the expression level for the cell line grown in vitro is also shown for comparison purposes. The expression of EGFR and KGFR transcripts was analyzed by quantitative reverse transcription-PCR (see Materials and Methods) and normalized relative to the housekeeping gene β-actin. Transcript expression levels are shown as a fold change relative to the expression by A427 cells used as a reference.
antibody cetuximab. Carcinoma cells were implanted 9 to 10 days before treatment began and dosed as shown in Fig. 3B. As expected, there was significantly less tumor growth in animals treated with the combination of cisplatin and cetuximab than in animals treated with vehicle (\(P < 0.0001\)) and the addition of palifermin had no effect on the antitumor efficacy of this combination (\(P = 0.7730\); Fig. 3B). Similar findings were observed in the FaDu xenograft model when another anti-EGFR antibody (panitumumab) was combined with palifermin and cisplatin (data not shown).

In these experiments, the tumor growth in animals treated with palifermin alone was similar to that seen in animals treated with vehicle (\(P > 0.9999\); Fig. 3B), showing no effect of palifermin on tumor growth in vivo.

**Effects of Palifermin in Combination with 5-FU and Bevacizumab on the Growth of HT29 Human Colon Carcinoma Xenografts**

Xenografts of HT29 carcinoma cells were implanted subcutaneously in nude mice and allowed to grow for 14 days before treatments were started. Palifermin was given for 3 days before and after 5 days of dosing with 5-FU. Bevacizumab was dosed twice a week for the duration of the experiment (Fig. 5A). In the animals treated with 5-FU and/or bevacizumab, tumor growth was significantly less than that seen in vehicle-treated animals (\(P < 0.0001\); Fig. 5A). The addition of palifermin to either 5-FU or bevacizumab treatment had no effect on the antitumor activity of these agents (\(P = 0.9991\)). Similarly, the addition of palifermin to the combination of 5-FU and bevacizumab also had no effect on antitumor activity (\(P = 0.8861\); Fig. 5A).

In animals treated with palifermin alone, HT29 tumor growth was similar to that seen in animals treated with vehicle (\(P > 0.9999\)), indicating that palifermin did not promote tumor growth in this model (Fig. 5A).

**Effects of Palifermin in Combination with 5-FU and Anti-EGFR Therapeutic Antibodies on the Growth of HT29 Human Colon Carcinoma Xenografts**

In these experiments, HT29 carcinoma cells were implanted 9 to 11 days before the start of treatment and the dosing regimens followed the same pattern as described above (Fig. 5B and C). When used alone, both 5-FU and cetuximab significantly decreased tumor growth compared with vehicle-treated animals (\(P < 0.0001\) and \(P = 0.0318\), respectively) and the antitumor efficacy of these agents was unaffected by the addition of palifermin. There was no statistically significant difference in tumor growth in animals treated with 5-FU or cetuximab alone compared with either of these agents combined with palifermin (\(P = 0.9985\); Fig. 5B). The combination of 5-FU and cetuximab led to a significant reduction in tumor size (\(P < 0.0001\)) and the efficacy of
this combination was unaffected by the addition of palifermin ($P = 0.9883$). Animals treated with panitumumab in combination with 5-FU also had significantly less tumor growth than did vehicle-treated animals ($P < 0.0001$; Fig. 5C). As was observed for cetuximab, the efficacy of the combination of panitumumab and 5-FU was unaffected by the addition of palifermin ($P = 0.9851$).

In both of these xenograft series, the tumor growth in animals treated with palifermin alone was similar to that seen in animals treated with vehicle ($P = 0.9995$ and 0.9545, respectively; Fig. 5B and C).

**Discussion**

There is a strong unmet need for more effective treatments for chemotherapy-induced mucositis in patients with a wide range of cancer types (21, 56), including head and neck (18, 19) and colorectal cancer (16). The pain and discomfort of mucositis in cancer patients can interfere with speech, the intake of food and medication, and overall quality of life (4, 57-59). It creates an avenue for infection (2), lengthens hospital stays, increases the need for parenteral narcotics and nutrition (2), and can interfere with anticancer treatment regimens (11, 56). A meta-analysis from 2004 found that among patients treated with standard-dose chemotherapy regimens who developed grade 3 to 4 mucositis, 70% required feeding tubes to maintain adequate nutrition, ~60% had fever, and 62% required hospitalization (11). This same analysis found that 35% of patients with grade 3 to 4 mucositis experienced delays in their next cycle of chemotherapy, 60% had to have their dose reduced, and 30% had to have their treatment discontinued altogether (11). In 2006, a study of patients with head and neck carcinoma found that unplanned interruptions or delays in chemotherapy occurred in 25% of patients with severe mucositis; 47% of these patients

![FIGURE 3.](image-url)
Finding from this study was that palifermin did not protect (HT29) carcinoma xenograft models. The most important these agents in human head and neck (FaDu) and colorectal colorectal cancer had any effect on the antitumor activity of and panitumumab) currently used to treat head and neck or and cisplatin) or biological agents (bevacizumab, cetuximab, combining palifermin with different chemotherapeutic (5-FU any safety signals.

In cancer patients who will have to be carefully monitored for reductions secondary to chemotoxicity (27, 54, 55). Conse- to improve patient comfort and functioning, to decrease the length of hospital stays, and to reduce the need for chemotherapy dose reductions secondary to chemotoxicity (27, 54, 55). Conse- quently, there is a strong desire to investigate the use of palifermin in patients receiving cancer therapy for nonhemato- logic malignancies such as colorectal and head and neck cancer. These investigations must proceed with care, however, because certain solid tumors express KGFRs (30), leading to a theoretical concern that a KGF-based therapy, such as palifermin, may promote tumorigenesis or protect tumors from the effects of anticaner agents. Preclinical studies can only provide limited guidance into the use of palifermin in solid tumor settings and they cannot unequivocally predict outcomes in cancer patients who will have to be carefully monitored for any safety signals.

The present study was designed to determine whether combining palifermin with different chemotherapeutic (5-FU and cisplatin) or biological agents (bevacizumab, cetuximab, and panitumumab) currently used to treat head and neck or colorectal cancer had any effect on the antitumor activity of these agents in human head and neck (FaDu) and colorectal (HT29) carcinoma xenograft models. The most important finding from this study was that palifermin did not protect FaDu human hypopharyngeal or HT29 human colon xenografts from the antitumor effects of either cytotoxic (5-FU or cisplatin), anti—vascular endothelial growth factor (bevacizu- mab), or anti-EGFR (cetuximab or panitumumab) therapies or from various combinations of these drugs used together. Palifermin also did not promote tumor growth in either model when it was used alone despite the fact that both of these carcinoma xenograft models expressed functional KGFRs. Moreover, despite the lack of an effect on tumor cells, palifermin did produce dramatic increases in esophageal thickness, showing that it was biologically active in these animal models. Despite these effects on normal epithelial cells, there was no evidence of enhanced tumor growth. This suggests that any potential effects that palifermin might have had on the tumor stroma did not result in any increased tumor cell growth. If palifermin had favored tumor growth by an indirect effect on the tumor microenvironment, these effects would have likely been observed in our experiments in the control groups that were treated with palifermin alone and/or any other treatment groups that included the combination with palifermin. That was never observed, suggesting that under the current experimental conditions, palifermin did not stimulate tumor cell growth either directly or indirectly through the stroma.

In conclusion, the results of the present study are supported by earlier in vitro and in vivo animal studies that showed that several tumor cells that express functional KGFRs exhibit little or no response to KGF (30). For example, in an HT29 xenograft model similar to the one used in the present study, KGF had no effect on tumor growth and did not protect the xenograft from the cytotoxic effects of 5-FU (25). Moreover, in a small preliminary dose-escalation study of the effects of palifermin on patients treated with the combination of 5-FU and leucovorin for metastatic colorectal cancer, palifermin seemed to have no effect on the time to disease progression, overall patient survival time, or progression-free patient survival time (27).

In conclusion, the results of the present preclinical study showed that palifermin did not promote the growth of human colon or hypopharyngeal xenografts and did not interfere with the antitumor efficacy of several different types and combina- tions of antitumor agents currently used to treat head and neck or colorectal cancers. The lack of an effect of palifermin on the efficacy of two different cytotoxic agents, two different anti-EGFR antibodies, and one anti—vascular endothelial growth factor antibody suggests that palifermin may have no drug interactions with other members of these drug classes. Nothing about the clinical safety or efficacy of palifermin in patients with head and neck or colorectal cancers can be inferred from the results of this animal study, but the results do suggest that further clinical investigations will be worthwhile and such studies are currently under way. At this time, however, palifermin should only be used in these patient populations during controlled clinical trials in which safety variables are carefully monitored both during and after the trial to ensure that palifermin did not have any negative effects on tumor outcomes.

**Materials and Methods**

**Assessing Expression of KGFR and EGFR Genes**

Twenty-two human carcinoma cell lines and 1 human glioblastoma cell line were obtained from the American Type
FIGURE 5. The effect of palifermin on the antitumor activity of 5-FU and various biological agents in mice bearing HT29 human colon carcinoma xenografts. A. HT29 model: effect of palifermin on 5-FU and bevacizumab. B. HT29 model: effect of palifermin on 5-FU and cetuximab. C. HT29 model: effect of palifermin on 5-FU and panitumumab. Tumor cells were implanted subcutaneously and allowed to grow for 9 to 14 d before treatments were started. The treatment protocols are indicated on the figure.
Culture Collection (ATCC). Cells were grown in standard 10% serum containing medium as outlined by the ATCC. Gene expression of KGFR and EGFR was assessed by quantitative reverse transcription-PCR (QRT-PCR) using the Taqman platform (Applied Biosystems). RNA was extracted using the RNeasy kit (Qiagen) and was then converted to cDNA using the Taqman cDNA kit (Applied Biosystems) using random prime oligonucleotides.

The expression of KGFR and EGFR transcripts was assessed using QRT-PCR along with a control PCR for β-actin gene expression. Primers and probes were designed using the Primer Express software (Applied Biosystems). Transcript expression levels were expressed relative to the A427 human lung carcinoma cell line. The SD for each test gene was expressed as a ratio relative to β-actin using the formula 2−ΔΔCt. To ensure that receptor expression was maintained during in vitro growth, QRT-PCR analyses on representative tumor samples excised from tumor xenograft–bearing animals were processed and analyzed in a similar way.

In vitro Signal Transduction

The FaDu human hypopharyngeal carcinoma and HT29 human colon carcinoma cell lines were also analyzed for their ability to initiate signal transduction when stimulated by KGF or EGF in vitro. In this analysis, 1 × 10⁶ cells were serum starved for 48 h before treatment with either KGF or EGF at 100 ng/mL, a pharmacologic relevant dose of KGF (palifermin) in humans (61, 62).

At various times between 5 and 90 min, the medium was removed and cells were washed, scrapped, centrifuged, and lysed in NP40 lysis buffer. The protein lysate was then assayed for phosphoprotein signal using the Meso-Scale Discovery kits to assess pS473 on the Akt protein or the pT202, pY204, pT185, pErk1, and pY187 sites on the Erk kinase. The phosphorylation levels were expressed relative to the growth, in vivo expression. Primers and probes were designed using the Primer system were supplied ad libitum. Room temperature was maintained between 68° F and 72° F, and relative humidity was maintained between 34% and 73%. The laboratory housing the cages provided a 12-h light cycle and met all International Association for the Assessment and Accreditation of Laboratory Animal Care specifications. The protocols were done at the Amgen, Thousand Oaks site, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

The FaDu and HT29 cell lines were originally obtained from the ATCC. The cells were maintained and expanded under routine tissue culture conditions at 37 °C in DMEM high glucose/5% fetal bovine serum, 1× nonessential amino acids, and 2 mM/L L-glutamine (Life Technologies). Cells were generally >90% viable at the point of transplant. Mice between 4 and 8 wk of age were challenged by subcutaneous injection of ~1 × 10⁶ cells per mouse in a 0.2 mL volume over the left flank. Nine to 14 d thereafter, when tumors had reached between 200 and 400 mm³ in volume, animals were randomized into groups (n = 10 per group) and treatment was begun as illustrated in Figs. 3 and 5. Tumor volumes as established by caliper measurements (length × width × height) were recorded twice per week, along with body weights as an index of toxicity. All experiments were conducted in a blinded fashion in which the individuals making the tumor measurements were unaware of the treatment that the animals had received.

Histology

For all tumor xenograft studies, four mice from the vehicle-treated group and four mice from the palifermin-treated group were sacrificed 4 d after the initiation of treatment. The esophagus was excised, fixed for 24 to 36 h in Z-FIX (3.7% formaldehyde, ionized zinc, in buffer; Anatech), and embedded into paraffin blocks using a Tissue Tek VIP paraffin tissue processor (Sakura USA). Sections (4 μm thick) were cut and mounted on Superfrost Plus slides (Fisher Scientific). All sections were deparaffinized, rehydrated, and stained with H&E (Lerner Laboratories). Sections were viewed with a Nikon FXA upright compound microscope using a 20× PlanApo Nikon lens. Images were recorded with a Nikon DXM1200 digital camera under standard bright-field Kohler illumination.

Reagents

Palifermin, panitumumab, human IgG2, and human IgG1 were provided by Amgen. Bevacizumab and cisplatin were obtained from Burt’s Pharmacy. Cetuximab was obtained from Vertrieb (distribution: Merck).

PBS was used as the dilution buffer for all study drugs. Palifermin (5 mg/kg) was administered subcutaneously in a dosing volume of 0.25 mL; antibodies and 5-FU were administered i.p. at the indicated dose in a 0.2 mL volume. For treatment with cisplatin, mice were weighed and dosed according to body weight; therefore, the exact volume of administration (10 μL/g) was variable but ~0.2 mL. To control for the effects of injections alone, all animals in each individual study received the same number of injections according to the same dosing schedule. If the animals in a particular treatment group were not designated to receive a particular study drug, they were treated with vehicle or IgG1 instead according to the same dosing schedule; human IgG1 was used as the control for bevacizumab and cetuximab; human IgG2 was used as the control for panitumumab.

Data Analysis and Statistical Methods

Data were expressed as means ± SE and plotted as a function of time. The statistical significance of observed differences between tumor growth curves was evaluated by repeated measures ANOVA followed by Scheffe post hoc testing for multiple comparisons. All statistical calculations were made through the use of StatView software v5.0 (SAS Institute, Inc.).

Disclosure of Potential Conflicts of Interest

All the authors are employees of Amgen, Inc., with the exception of Suijin Yang who was employed by Amgen, Inc. at the time of the study, but who is now affiliated with Pfizer, Inc. No other potential conflicts of interest were disclosed.
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References


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