Preclinical Characterization of CG53135 (FGF-20) in Radiation and Concomitant Chemotherapy/Radiation-induced Oral Mucositis

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ABSTRACT

Purpose: The purpose of this study was to evaluate the activity of CG53135 (FGF-20), a protein with in vitro mitogenic activity on epithelial and mesenchymal cells, in two in vivo models of oral mucositis (OM).

Experimental Design: Radiation or concomitant chemotherapy/radiation-induced OM was elicited in hamsters. Activity of CG53135 was assessed at different doses and regimens in the models. Bromodeoxyuridine (BrdUrd) incorporation and pharmacokinetic studies were also performed to correlate in vivo activity of CG53135 with exposure.

Results: In the hamster radiation model, administration of CG53135 (600 or 1200 μg/day, i.p.) on days 3–15 resulted in a statistically significant (P < 0.001) reduction in days spent with severe mucositis. CG53135 administered at 12 mg/kg, i.p. (days 1–2 or 1–8) in the concomitant chemotherapy/radiation model resulted in a statistically significant (P < 0.001) reduction in severe mucositis. Maximal BrdUrd incorporation was observed in cheek pouch and jejunal tissues at 8 h, and peak plasma levels of CG53135 were reached 1 h after administration.

Conclusions: CG53135 demonstrates potent, regimen-dependent activity in hamster models of OM. The activity was regimen dependent. BrdUrd incorporation studies confirmed that CG53135 had proliferative activity in vivo with a favorable pharmacokinetic profile. Based in part on work described herein, CG53135 has received approval from the United States Food and Drug Administration to be evaluated in a Phase I clinical trial of cancer patients at risk for developing OM.

INTRODUCTION

OM1 is a common, painful, dose-limiting toxicity associated with both radiation and chemotherapy. OM, especially in its ulcerative form, increases morbidity, mortality, and cost of current cancer therapeutic modalities (1–4). The presence of the ulcerative OM lesions in a patient population compromised by granulocytopenia often leads to oral and systemic infections. It has been recognized that OM may significantly impact the quality of life of patients undergoing radiation or chemotherapy (5). Furthermore, OM is sometimes cause for clinicians to interrupt radiation and chemotherapeutic protocols in oncology patients, thus limiting the success of therapeutic plans. Current OM therapies are mostly palliative. Some of these therapies include mucosal barrier agents, antiseptics, antibiotics, analgesics, and general oral hygiene (6, 7). In the quest for a more active approach, a number of biological agents, including transforming growth factor-β3, granulocyte macrophage colony-stimulating factor, interleukin-11, and keratinocyte growth factor-1 (or FGF-7) have been evaluated for activity against OM (8–14).

The oral mucosa is complex tissue composed of diverse cellular populations, including renewing stratified squamous epithelium, basal epithelial stem cells, connective tissue fibroblasts, endothelium, and at times, an inflammatory infiltrate that is of changing composition. Mechanistically, OM has been described as having four phases (inflammation/vascular, epithelial, ulcerative/bacterial, and healing/resolution) that are initiated by the delivery of radiation or chemotherapy (15, 16). Mediators involved in the progression of OM remain under active investigation. A widely held hypothesis regarding the underlying mechanism of mucositis postulates that radiation or chemotherapy triggers a cascade-like response between the different cellular and extracellular components that comprise the oral mucosal tissue. It is the complex interplay between epithelial microenvironment, extracellular components, and extrinsic factors that leads to clinical manifestation of OM. Although initial hypotheses describing OM pathophysiology placed a large emphasis on the role of mucosal epithelial injury, current research has been focusing on other mucosal cellular components, including connective tissue fibroblasts, endothelium, and inflammatory infiltrate as important mediators in development and outcome of OM (17, 18). Previous work has already established that mesenchymal cells and extracellular interactions play important roles in developing epithelium (19).

1 The abbreviations used are: OM, oral mucositis; FGF, fibroblast growth factor; Gy, Gray; CCR, concomitant chemotherapy/radiation; BrdUrd, bromodeoxyuridine; 5-FU, 5-Fluorouracil.

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2 The abbreviations used are: OM, oral mucositis; FGF, fibroblast growth factor; Gy, Gray; CCR, concomitant chemotherapy/radiation; BrdUrd, bromodeoxyuridine; 5-FU, 5-Fluorouracil.
In the context of OM, the FGF family presents a logical step in the development of therapeutic agents to treat this condition. FGF-7 is mitogenic to epithelial cells and has shown activity in preclinical models of epithelial ulceration (20, 21). CG53135 (FGF-20) is a recently described member of the FGF family that has mitogenic activity on epithelial and mesenchymal cells (22). A recent study showed that CG53135 confers potent intestinal mucosal protection in animal models of ulcerative colitis (23), suggesting its potential in treatment of other forms of mucosal damage. The present work describes the effect of CG53135 as an active agent against OM, in well-defined in vivo models of radiation and CCR-induced OM (24, 25).

MATERIALS AND METHODS

Animals. Five- to 6-week-old male Golden Syrian hamsters (Charles River Laboratories, Wilmington, MA) weighing 80–90 grams were used throughout the study. All protocols complied with the Massachusetts College of Pharmacy Institutional Animal Care Committee Guidelines and guidelines presented by the Guide for the Care and Use of Laboratory Animals. Animals were individually identified and maintained in temperature- and light-controlled rooms (12-h cycle). Animals had access to food and water ad libitum. Groups in all mucositis studies consisted of 8–10 animals.

Protein Purification. Recombinant human CG53135 (FGF-20) was purified from Escherichia coli BLR (DE3) cells (Novagen, Darmstadt, Germany). Briefly, DE3 cells were transformed with full-length, codon-optimized CG53135 cDNA cloned in a pET24a vector (Novagen). A manufacturing cell bank of these cells was produced. After fermentation and induction, cell paste containing CG53135 protein was lysed with high-pressure homogenization in lysis buffer and clarified by centrifugation. CG53135 was purified from the clarified cell lysate by two cycles of ion exchange chromatography and ammonium sulfate precipitation. The final precipitate was washed with purified water and resolubilized in formulation buffer. Human recombinant CG53135 was >95% pure as determined by SDS-PAGE followed by Coomassie Blue staining.

Acute Radiation-induced Mucositis. OM was induced in hamsters by a single administration of 40 Gy. On day 0, under anesthesia (pentobarbital, 60 mg/kg, i.p.), the left buccal pouch was everted, while the rest of the individual animal was shielded with lead. Radiation was generated using a 160-kV potential (18.75-ma) source (Gemini X-ray Irradiator; Yxlon International, Inc., Akron, OH), at a focal distance of 21 cm, hardened with a 3-mm Al filtration system. Ionizing radiation was specifically targeted at the left buccal pouch mucosa at a rate of 1.32 Gy/minute. Anesthetized animals were monitored during recovery and returned to their cages. In this model, mild reversible weight loss is noted, and no other systemic effects are elicited in the animals because of direct targeting of the radiation field. CG53135 was administered at 300, 600, or 1200 μg/day, i.p., on days 3–15. For reference, the 600 μg/day dose corresponded to an average of 7.2 mg/kg/day (ranging from 8 to 6.7 mg/kg/day over the duration of the study). A control group consisted of irradiated animals dosed with vehicle on days 3–15. This dosing regimen was designed to cover the period of OM characterized by the late inflammatory, epithelial, and ulcerative phases of the condition.

CCR-induced Mucositis. CCR-induced mucositis was elicited in hamsters by administration of two doses of 5-FU (Sigma, St. Louis, MO; 60 mg/kg, i.p.) on days −4 and −2. On day 0, all animals were anesthetized (pentobarbital, 60 mg/kg, i.p.), their left buccal pouches were everted, and a single dose of radiation (30 Gy) was administered. CG53135 was administered at 600 or 1200 μg/day, i.p. on days −5 to 18 or days 1–18 (mean daily dose of 6 or 12 mg/kg). A similarly induced CCR study was dosed with CG53135 (12 mg/kg/day, i.p.) on days 1–2, 1–9, 1–18, or 6–14. Dosing schedules covering several time periods before and after OM induction were performed to define an optimal dosing schedule for CG53135 in the CCR OM model.

Mucositis Evaluation. The progression of mucositis was monitored daily for both models. Every other day starting on day 6 postirradiation, animals were anesthetized using inhalation anesthesia, and the left buccal pouch was everted and photographed. At the conclusion of the study’s clinical phase, film was developed, and resulting photos were randomly numbered and then scored in blinded fashion by two observers. A 0–5 scoring system was used that applied the following numerical score to buccal lesions: 0, normal mucosa; 1, erythema and vasodilation; 2, severe erythema and vasodilation, with erosion of superficial aspects of mucosa leaving denuded areas with decreased stippling of mucosa. 3, severe erythema, vasodilation, and formation of ulcers in one or more places. Cumulative size of ulcers involved ~25% of the pouch mucosa. Pseudomembrane formation is evident. 4, severe erythema and vasodilation. Cumulative size of ulcers involved about half of the pouch mucosa. Loss of mucosal pliability. 5, diffuse, extensive ulceration. Loss of pliability, pouch can only partially be extracted from mouth. The reported scores represent the average of the observations from the two blinded observers. In this model, a score of ≥3 coincides with a clinically significant National Cancer Institute or WHO score ≥3.

Severity of OM was calculated using the scores per treatment group on each observation day (mean ± SE). Using the severity scores, results are also represented as percentage of days with a score of three or above. The percentage of animal days reflects the overall duration of severe mucositis for each group (as days ≥3) over the total number of days observed. χ² analysis for significance was performed to evaluate the differences between the groups. A score was considered significantly different if a χ² analysis demonstrated a probability value of <0.05.

BrdUrd Incorporation Studies. Normal hamsters received a single dose of CG53135 (12 mg/kg, i.p.) at time 0. At 0, 2, 6, and 22 h after injection, each animal received a single dose (50 mg/kg, i.p.) of BrdUrd. Two h later, three animals per time point were euthanized. Cheek pouch and jejunum tissues were collected at necropsy. Untreated control animals were similarly treated and examined 2 h after receiving BrdUrd. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, and mounted. Using a biotinylated anti-BrdUrd monoclonal antibody (Oncogene Research Products, San Diego, CA), the tissues were stained to identify nuclear BrdUrd incorporation. All tissues were additionally counterstained with hematoxylin and evaluated in blinded fashion by
two different observers. Ten high-power fields were evaluated in two sections of each sample. The reported results reflect the mean number of stained cell nuclei (±SE) per high-power field in each experimental group.

Pharmacokinetic Profiling Studies. CG53135 was administered as a single bolus dose (6 mg/kg, i.p.) at time 0. After a period of 1, 2, 4, 8, or 24 h, hamsters were euthanized and exsanguinated. All blood was collected in EDTA-containing tubes for plasma separation. Plasma was stored at −80°C until further processed. Plasma concentrations of CG53135 were determined by a sandwich ELISA. Briefly, 96-well plates were coated with 5 μg/ml monoclonal antibody (raised against an NH₂-terminal peptide of CG53135) overnight at 4°C. The wells were blocked (1 h at room temp) with PharMingen dilution buffer (San Diego, CA) and washed in 0.05% Tween 20 in PBS. Plasma samples were diluted 10-fold in PharMingen dilution buffer and then added to the wells. Bound CG53135 was detected with 5 μg/ml rabbit anti-CG53135 polyclonal antibody (produced by Rockland Immunochemicals, Inc. using CG53135 for immunization). After the addition of horseradish peroxidase-conjugated donkey antirabbit immunoglobulin and subsequent addition of 3, 3’, 5, 5’-tetramethylbenzidine reagent, and stop solution (17% phosphoric acid), absorbance was read at 450 nm. Absorbance readings were then converted (using SoftMax Pro 3.1.2) to protein concentration values by comparison with a standard curve consisting of known amounts of CG53135 added to an equivalent dilution of plasma as the test samples. Five animals were used for each time point evaluated. Pharmacokinetic parameters were analyzed by noncompartmental methods using PK Solutions 2.0 (Summit Research Services, Montrose, CO).

RESULTS

**Radiation-induced Mucositis.** The effect of CG53135 on acute radiation-induced mucositis was first studied. In vehicle-treated animals, mucositis peaked on day 16 with a mean score of 3.2 ± 0.1, retreating to 2.8 ± 0.3 on day 28 (Fig. 1A). A modest effect of CG53135 at 300 μg/day (days 3–15) was observed. Peak mean mucositis occurred on day 18 (3.2 ± 0.1), and by day 28, the mean mucositis score was 2.3 ± 0.4. In contrast, treatment with CG53135 at 600 μg/day (days 3–15) reduced the peak mean mucositis score to 2.8 ± 0.2 on day 16, which diminished to 2 ± 0.3 by day 28. Similarly, administration of CG53135 at 1200 μg/day (days 3–15) resulted in a peak mean mucositis score of 2.7 ± 0.3 on day 16 and more rapid resolution than noted in the other groups, ending with a score of 2.1 ± 0.4 on day 28.

Duration of mucositis was evaluated by calculating the percentage of animal days spent with a score of ≥3 for each group. Vehicle-treated animals spent 68% of animal days with scores of ≥3 (Fig. 1B). CG53135 reduced the duration of severe OM. Hamsters treated with CG53135 at a dose of 300 μg/day (days 3–15) spent 57% animal days with score of ≥3. Treatment with 600 μg/day reduced the total days with a score ≥3 to 41% and treatment with 1200 μg/day further reduced scores to 31%. χ² analysis shows the difference between treatment with CG53135 (600 and 1200 μg/day) to be statistically significant (P < 0.001) when compared with vehicle-treated controls.

**CCR-induced Mucositis.** The effect of CG53135 was next assessed in a mucositis model induced by CCR. Compared with the previous model, CCR mucositis development is dependent on treatment with 5-FU and uses a lesser amount of radiation to trigger local mucositis within the buccal pouch. The individual use of radiation or 5-FU at these doses is not able to elicit a significant OM in this species. Vehicle-treated animals (days 5–18) reached peak mean mucositis on day 16 (3.2 ± 0.1), which decreased to 2.3 ± 0.3 by day 30 (Fig. 2A). These animals spent 53% of animal days with a score ≥3 (Fig. 2B). The effect of CG53135 on OM in the CCR model was markedly influenced by both dose and dosing schedule. When CG53135 was administered (600 or 1200 μg/day) from days 5 to 18, mean group mucositis peaked on day 16 at 3.2 ± 0.1 and 3.4 ± 0.2, respectively. By day 30, animals dosed with either 600 or 1200 μg/day had scores of 2.8 ± 0.1 or 2.7 ± 0.2, respectively. These groups spent 65 and 77%, respectively, of animal days with severe mucositis (Fig. 2B). Administration of CG53135 with 1200 μg/day from day 5 to 18 contributed to a significant worsening of OM when compared with vehicle-treated control animals (P < 0.001).
However, when the schedule was modified for later treatment (days 1–18), CG53135 administered at comparable doses (600 or 1200 μg/day) elicited a potent treatment response. Animals treated with CG53135 (600 or 1200 μg/animal/day, i.p.) on days 5–18 or 1–18 and mean mucositis scores (A), as well as percentage of animal days with a score ≥ 3 (B) were assessed. –△–, vehicle treated; –○–, 600 μg/days 5–18; –□–, 1200 μg/days 5–18; –■–, 600 μg/days 1–18; –○–, 1200 μg/days 1–18.

To determine an optimal dosing schedule in the CCR model, CG53135 was dosed at 12 mg/kg/day using four different regimens (Fig. 3). In this experiment, vehicle-treated animals reached a peak mucositis score of 3.3 ± 0.1 on day 16, which diminished to 2.6 ± 0.2 by day 30 (Fig. 3A). Vehicle-treated animals spent a total of 61% animal days with severe mucositis (Fig. 3B). Hamsters treated with CG53135 at 12 mg/kg daily (days 1–18) had a peak mean mucositis score of 2.9 ± 0.2 on day 14, which was reduced to 2.4 ± 0.2 by day 30. Similarly, hamsters treated with 12 mg/kg on days 1–2 showed a mean peak mucositis score of 2.8 ± 0.2 on day 16, which was reduced to 2.1 ± 0.2 by day 30 (Fig. 3A). Animals treated from days 1 to 18 or 1 to 2 spent a total of 37 and 28% animal days with severe mucositis, respectively (Fig. 3B). For both schedules, these results constitute a statistically significant difference when compared with vehicle-treated animals (P < 0.001). In contrast, animals dosed (12 mg/kg/day) on days 6–14 or 1–9 had mean peak mucositis scores of 2.9 ± 0.1 and 3.3 ± 0.2, respectively (Fig. 3A). Scores at day 30 for the 6–14 and 1–9 groups were 2.1 ± 0.2 and 2.4 ± 0.2, respectively (Fig. 3A). Hamsters treated on days 6–14 or 1–9 spent a total of 48 and 63%, respectively, of animal days with severe mucositis (Fig. 3B).
The treatment effect on days 6–14 was statistically significant when compared with vehicle control animals ($P = 0.018$).

**BrdUrd Labeling Studies.** To determine if CG53135 induces proliferative activity in vivo, BrdUrd incorporation in mucosal tissues was assessed 0, 2, 4, 8, or 24 h after a single injection of CG53135 (12 mg/kg, i.p.). Untreated control cheek pouch epithelium showed an average of 1 ± 0 (mean ± SE) stained nuclei/field (Fig. 4, A and B), whereas CG53135-treated animals had 1.6 ± 1 stained nuclei/field at 2 h (Fig. 4, A and C), declining to 0 after 4 h (Fig. 4, A and D). An increase of BrdUrd incorporation was observed at 8 h when cheek pouch epithelium showed an average of 4 ± 3 stained nuclei/field (Fig. 4, A and E). At 24 h, no stained nuclei/field were present (Fig. 4, A and F). A similar temporal pattern of BrdUrd incorporation in jejunal tissues was found when compared with cheek pouch staining. A slight elevation of BrdUrd incorporation was observed at 2 h, followed by a decline at 4 h, peak incorporation at 8 h, and low levels at 24 h (Fig. 5, A–F).

**CG53135 Pharmacokinetics.** The plasma pharmacokinetic behavior of CG53135 was evaluated after a single bolus dose (6 mg/kg, i.p.). Average peak plasma concentration of 290 ng/ml was reached by 1 h (Fig. 6). Plasma concentrations of CG53135 declined steadily thereafter with a terminal elimination half-life of 20 h. The area under the curve was 1210 ng/h/ml. As blood collections were performed as terminal exsanguinations rather than as serial sampling of individual animals, it was not possible to determine the statistical ranges of the calculated pharmacokinetic parameters.

**DISCUSSION**

OM remains a significant clinically unmet challenge associated with cancer therapy. Our understanding of the underlying mechanisms for this condition has been steadily increasing over the last decade; however, the cellular and molecular mediators...
in OM remain incompletely defined. To assist in its mechanistic characterization, OM has been divided into distinct phases based on the predominant biological features present at specific times of histopathological observation. These phases of OM (inflammation/vascular, epithelial, ulcerative/bacterial, and healing/resolution) are sequential in their manifestation, with a degree of overlap. As these steps progress from induction through resolution, the mucosal microenvironment is in a state of dynamic change. An understanding of this process has allowed for the development of targeted biological therapeutic agents for the management of OM. CG53135 (FGF-20) possesses mitogenic in vitro activity toward fibroblasts and epithelial cells and has demonstrated potent in vivo activity in animal models of inflammatory bowel disease (22, 23). In this study, CG53135 activity was assessed in validated hamster models of mucositis. In addition, experiments were performed to elucidate the phase(s) in the mucositis process affected by CG53135.

Significantly, CG53135 administered from day 3 to 15 after an acute dose of ionizing radiation reduced the severity and duration of OM. This schedule assessed the activity of CG53135 during the late inflammatory phase through the epithelial, ulcerative, and early healing phases and established a dose range for CG53135. Evaluation of CG53135 activity in the CCR-induced model of OM demonstrated the importance of dose scheduling on the efficacy of this agent. Administration of CG53135 before OM induction through early healing phase failed to reduce the severity or course of OM compared with controls and in fact made OM significantly worse. In contrast, treatment initiated immediately after OM induction significantly reduced both the severity and duration of OM.

Further evidence of the importance of dose scheduling and duration of dosing was demonstrated when other schedules were used in the same model. Dosing of hamsters with CG53135 on days 1–2 or 1–18 significantly reduced both the severity and duration of mucositis. However, administration on days 6–14 was less efficacious, and administration on days 1–9 was non-
efficacious. We hypothesize that this variability in outcome can be attributed to multifactorial elements in the progression of mucositis intrinsic to the various stages observed in the process. Because the composition of this microenvironment changes on a temporal basis, the observed response of the tissue may change because cellular components and mediators vary over time. This phenomenon has been illustrated through ongoing transcription profiling studies in the model where differential expression of several potentially important proteins has been shown to be temporal in nature (18). Thus, finding the correct dose and optimal dosing schedule may be essential to demonstrate activity of CG53135 in clinical OM.

In the present study, CG53135 showed an ability to stimulate cell proliferation in vivo when administered at a dose of 12 mg/kg. The temporal pattern of BrdUrd incorporation was similar for both cheek pouch mucosa and jejunal epithelium. Generally, there is a reduction in BrdUrd incorporation at 4 h, followed by peak incorporation level at 8 h. This peak is reduced to basal concentration or below at 24 h. The increased DNA synthesis observed after CG53135 administration confirmed that this agent induces epithelial proliferation in vivo and defines two CG53135-responsive target tissues, cheek mucosa and jejunum epithelium.

The plasma pharmacokinetic profile of CG53135 demonstrates peak levels at ~1 h and detectable levels (low ng/ml) up to 24 h after administration. In vitro experiments on a variety of cell types have indicated that CG53135 is mitogenically active at this range (22). Thus, the observed pharmacokinetic profile is consistent with an interpretation that CG53135 exposure levels are sufficient to account for in vivo BrdUrd and OM activity results.

In conclusion, CG53135 is an effective agent in reducing both the severity and duration of OM in vivo. The positive effects of CG53135 are clearly demonstrated in a radiation and CCR-induced OM model. CG53135 appears to act by temporal stimulation of epithelial cell proliferation in the oral mucosa. Although it is likely that the direct mitogenic activity on epithelial cells is responsible for the marked improvement in mucositis scores, it is also possible that effects on mucosal mesenchymal cells lead to further epithelial repair. Protection of both oral and intestinal epithelium could provide a better general outcome in cases of systemic administration of stomatotoxic agents. Additional work is under way to further characterize the mechanistic properties of CG53135 in epithelial tissues.

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