Cancer Chemother Pharmacol (2002) 50: 202–212
DOI 10.1007/s00280-002-0493-8

ORIGINAL ARTICLE

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In vitro and in vivo effects of repifermin (keratinocyte growth factor-2, KGF-2) on human carcinoma cells

Received: 31 August 2001 / Accepted: 7 June 2002 / Published online: 30 July 2002

Abstract *Purpose:* Repifermin (keratinocyte growth factor-2, KGF-2) is a growth factor that selectively induces epithelial cell proliferation, differentiation and migration. The objective of this study was to assess the effect of repifermin on in vitro tumor cell proliferation and in vivo tumor growth using a variety of human carcinoma cell lines with differing growth rates and levels of KGF receptor (KGFR) expression. *Methods:* Potential effects of repifermin on in vitro cell proliferation were evaluated by alamarBlue and/or [^{3}H]-thymidine incorporation assays under a range of serum conditions. In vivo tumor growth was evaluated by implanting KGFR$^+$ carcinomas subcutaneously into nude mice and measuring tumor growth over time in mice injected intravenously (i.v.) or intraperitoneally (i.p.) with repifermin or placebo. *Results:* In vitro, none of the 30 human carcinoma cell lines tested demonstrated a substantial increase in proliferation in response to repifermin over the concentration range 0.01 to 1000 ng/ml. In vivo results showed no significant tumor growth-promoting activity when single- or multiple-cycle intravenous injections of repifermin (1 mg/kg) were given to athymic nude mice inoculated with human KGFR$^+$ tumors of the pharynx (Detroit 562, FaDu), colon (Caco-2), salivary gland (A-253) or tongue (SCC-25, CAL 27). In addition, repifermin (0.2 or 2 mg/kg) injected i.p. for 2 weeks had no effect on the growth of eight other human carcinomas including those of the ovary (NIH:OVCAR-3, SK-OV 3, PA-1), bladder (SCaBER), epidermis (A 431), lung (SW 900), breast (MDA-MB-231) and cervix (SiHa). *Conclusions:* Repifermin had no in vitro or in vivo proliferative effects on KGFR$^+$ human epithelial-like tumors. This failure to stimulate tumor cell growth highlights the ability of repifermin to specifically target normal epithelial tissue. This is critical to the safety profile of repifermin, since it is currently in phase II clinical trials for the treatment of cancer patients with mucositis resulting from chemo- or radiotherapy.

Keywords Fibroblast growth factors · Human carcinoma · Keratinocyte growth factor · Repifermin · Tumor growth

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Introduction

Repifermin, a recombinant form of keratinocyte growth factor-2 (KGF-2) or fibroblast growth factor-10 (FGF-10), belongs to the FGF family of growth factors [6] and emerged from a genomics-based screening program. It binds to a splice variant of FGF-receptor type-2 (FGFR2iiib or KGFR) [10] as well as to the FGFR1iiib receptor. Among the various FGF growth factors, repifermin is most closely related to KGF-1 (FGF-7) [7], with which it shares 57% homology [6]. These two growth factors both recognize the FGFR2iiib receptor, and as a consequence induce epithelial cell proliferation [14, 23]. However, only repifermin binds to the FGFR1iiib receptor and induces proliferation of BaF3 cells transfected with that receptor [13]. How binding to dual FGF receptors affects the in vivo activity of repifermin is not yet clearly understood, although it has shown activity in several in vivo models of mucosal injury [8, 18]. It is currently in clinical trials as a potential therapeutic agent for the treatment of venous ulcers, ulcerative colitis and chemotherapy-/radiotherapy-induced mucositis.

Tissue distribution of FGFR2iiib, the receptor recognized by both keratinocyte growth factors, is restricted to cells of epithelial origin. A theoretical toxic side effect resulting from chronic treatment of patients with repifermin could be induction of epithelial derived tumor growth. The receptor is expressed on a variety of

malignant carcinoma cell lines [2, 5, 12], raising the possibility that repifermin might enhance the growth of these tumor lines. In a recent study, it has been shown that KGF-1 induces a weak in vitro proliferative response in the $KGFR^+$ squamous carcinoma cell lines SCC-9, SCC-25, FaDu, and Detroit 562 [20]. However, when KGF-1 was tested in vivo, it failed to show tumor growth-promoting activity against FaDu, Detroit 562, or A431 cells, the three $KGFR^+$ carcinoma cell lines tested [20]. As a critical component of the preclinical safety profile of repifermin, the studies described here were designed to assess the in vitro and in vivo effects of repifermin on proliferation of various human tumor cell lines with different levels of KGF receptor expression and growth rates.

Materials and methods

Keratinocyte growth factor-2

Repifermin (KGF-2) was expressed in *Escherichia coli*, purified, and formulated by Human Genome Sciences as lyophilized material containing 7% sucrose, 10 m*M* Na citrate, 20 m*M* NaCl, and 1 m*M* EDTA. Repifermin was reconstituted in sterile water, resulting in a stock concentration of 10 mg/ml. A buffered vehicle containing the excipients used in the repifermin formulation was used to dilute the reconstituted repifermin.

Tumor and reference cells

The human tumor cell lines were supplied by the American Type Culture Collection (ATCC; Manassas, Virginia). The cell line number, tumor type, tissue source, and ATCC catalog number for each of these cell lines are provided in Tables 1, 2 and 3. Human primary keratinocytes were obtained from Clonetics Corporation (Walkersville, Md.).

KGFR (FGFR2iiib) expression in human tumor cells

Expression levels of KGFR in human tumor cell lines and in normal human primary keratinocytes were determined by a real-time quantitative polymerase chain reaction (PCR) procedure using the ABI Prism 7700 Sequence Detection System and a TaqMan PCR Reagent Kit (Applied Biosystems, Foster City, Calif.). Amplification primers and probe were designed to span the region from nucleotide 1466 to nucleotide 1538 of the human KGFR sequence (Genbank accession no. M80634). Cells were harvested between passages two and four and the total RNA prepared using the Trizol extraction procedure according to the manufacturer's instructions. KGFR mRNA was detected by a one-step RT-PCR

Table 1. Summary of in vitro and in vivo proliferative effects of repifermin on human tumors associated with the oral cavity or alimentary tract. All tumor cell lines were ordered from ATCC. Receptor expression was evaluated using quantitative PCR and compared to the expression levels in primary human keratinocytes. Induction of in vitro proliferation by repifermin (0.01–10,000 ng/ml) was measured using alamarBlue staining and/or [^{3}H]-thymidine incorporation in medium containing 0.1% or 10% FBS. To quantify tumor growth in vivo, human tumors were implanted s.c., nude mice were injected i.v. with repifermin, and the tumor sizes (mm^2) were plotted over time

Cell line	Tumor cell type/ tissue source	ATCC no.	Receptor expression (compared to keratinocytes)	In vitro-enhanced proliferation	In vivo-enhanced growth
Keratinocyte	Human primary cells	–	1.0	+	
Detroit 562	Carcinoma/pharynx	CCL-138	8.2	–	–
Caco-2	Colorectal adenocarcinoma/colon	HTB-37	7.5	–	–
A-253	Epidermoid carcinoma/salivary gland	HTB-41	3.5	–	–
SCC-25	Squamous cell carcinoma/tongue	CRL-1628	1.5	–	–
CAL 27	Squamous cell carcinoma/tongue	CRL-2095	0.9	–	–
FaDu	Squamous cell carcinoma/pharynx	HTB-43	0.5	–	–

Table 2. Summary of in vitro and in vivo proliferative effects of repifermin on human tumors of varying origin and KGFR expression levels. All tumor cell lines were ordered from ATCC. KGFR expression on tumor cells was evaluated using quantitative PCR and compared against expression levels in primary human keratinocytes. Induction of in vitro proliferation by repifermin (0.01–10,000 ng/ml) was measured using alamarBlue staining in medium containing 0.1% or 10% FBS. To quantify tumor growth in vivo, human tumors were implanted s.c., nude mice were injected i.p. with repifermin, and the tumor sizes (mm^2) were plotted over time

Cell line	Tumor cell type/ tissue source	ATCC no.	Receptor expression (compared to keratinocytes)	In vitro-enhanced proliferation	In vivo-enhanced growth
Keratinocyte	Human primary cells	–	1.0	+	
NIH:OVCAR-3	Adenocarcinoma/ovary	HTB-161	11.0	–	–
SCaBER	Squamous cell carcinoma/bladder	HTB-3	3.5	–	–
A 431	Epidermoid carcinoma/epidermis	CRL-1555	2.0	–	–
SW 900	Squamous cell carcinoma/lung	HTB-59	0.1	–	–
SK-OV 3	Adenocarcinoma/ovary	HTB-77	0.0	–	–
PA-1	Teratocarcinoma/ovary	CR-1572	0.0	–	–
MDA-MB-231	Adenocarcinoma/breast	HTB-26	0.0	–	–
SiHa	Squamous cell carcinoma/cervix	HTB-35	0.0	–	–

Table 3. Summary of in vitro proliferative effect of repifermin on human colorectal adenocarcinomas with varying levels of KGFR expression. All tumor cell lines were ordered from ATCC. Receptor expression was evaluated using quantitative PCR, and KGFR expressed as a fraction of 18S ribosomal RNA. Induction of in vitro proliferation by repifermin (0.01–1000 ng/ml) was measured in terms of [^{3}H]-thymidine incorporation in the presence or absence of 5% FBS. Colon antigen expression levels were taken from the ATCC catalog [9] (*ND* not described)

Cell line	Cell type	ATCC no.	Carcinoembryonic antigen (ng/10^6 cells)	Colon-specific antigen	Colon antigen 3	Receptor expression (fraction of 18S ribosomal RNA)	In vitro-enhanced proliferation
LS 174T	Colorectal adenocarcinoma	CL-188	1944	+	ND	7×10^{-5}	–
SW1417	Colorectal adenocarcinoma	CCL-238	362	–	–	3×10^{-5}	–
SW48	Colorectal adenocarcinoma	CCL-231	0.6	–	ND	3×10^{-5}	–
SW480	Colorectal adenocarcinoma	CCL-228	0.7	–	–	1×10^{-5}	–
COLO 201	Colorectal adenocarcinoma	CCL-224	0	–	–	7×10^{-6}	–
SW403	Colorectal adenocarcinoma	CCL-230	155	–	+	4×10^{-6}	–
DLD-1	Colorectal adenocarcinoma	CCL-221	0.5	–	+	4×10^{-6}	–
LoVo	Colorectal adenocarcinoma	CCL-229	908	–	–	2×10^{-6}	–
SW837	Colorectal adenocarcinoma	CCL-235	523	–	–	1×10^{-6}	–
SW620	Colorectal adenocarcinoma	CCL-227	0.15	–	–	6×10^{-9}	–
SW948	Colorectal adenocarcinoma	CCL-237	6.9	+	ND	6×10^{-9}	–
COLO 320 DM	Colorectal adenocarcinoma	CCL-220	0	–	–	0	–

(reverse transcriptase PCR) procedure. For quantitation of KGFR mRNA, the comparative Ct method was used (Perkin Elmer User Bulletin no. 4, 1997), employing an 18S ribosomal RNA probe for normalization. The receptor expression level obtained for the keratinocytes was assigned a value of 1, and values relative to this were calculated for the tumor cell lines listed in Tables 1 and 2. Cell lines are listed in rank order from the highest to the lowest levels of KGFR message expression.

Additionally, as listed in Table 3, KGFR expression in a series of colorectal adenocarcinoma cell lines was quantified and expressed as a fraction of total 18S ribosomal RNA. In terms of approximate number of copies per cell, it is estimated that a fraction of 10^{-4} is equivalent to about 1000 copies, 10^{-5} about 100 copies, 10^{-6} about 10 copies, and 10^{-7} about 1 copy.

In vitro proliferation assays

The primary approach to assessing the in vitro proliferative effects of repifermin was the use of the vital dye alamarBlue [1]. Because fetal bovine serum (FBS) contains growth factors that stimulate the growth of cell lines of epithelial origin, it was essential to distinguish any proliferative effects of repifermin from those induced by FBS. Therefore, the 18 human carcinoma cell lines shown in Tables 1 and 2 were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.) with 0.1% FBS (Gibco). Cells were grown in 96-well plates (Corning, Corning, N.Y.) at a density of 3000 cells/well, with repifermin added at concentrations in the range 0.01–10,000 ng/ml. Serial dilutions of the vehicle were used as negative controls. Following incubation of cells at 37°C for 5 days, 10% alamarBlue was added, followed by an additional incubation at 37°C for 4 to 6 h. Fluorescence was read using a CytoFluor fluorescence reader (PerSeptive Biosystems, Framingham, Mass.) with an excitation wavelength of 530 nm and emission at 590 nm. The higher the absorbance, the greater the proliferation. To evaluate the effect of repifermin on tumor growth under optimal conditions, this assay was also conducted using complete (10% FBS) medium throughout.

In addition to the alamarBlue assay, [^{3}H]-thymidine incorporation was used to confirm proliferation of the representative tumor cell lines Caco-2, A-253, CAL 27, and HEp-2. Briefly, tumor cells were plated in a Corning 24-well plate (30,000 cells/well) with medium containing 10% FBS and were allowed to adhere overnight. The following day, growth medium was replaced with medium containing 0.1% FBS plus repifermin, diluted in formulation buffer at concentrations in the range 0.01–10,000 ng/ml. Cells were incubated for 24 h and pulsed on the following day with [^{3}H]-thymidine (1 μCi/well) for 5 h. The amount of [^{3}H]-thymidine incorporated into trichloracetic acid-precipitable material was determined using a beta scintillation counter (Beckman Coulter, Fullerton, Calif.).

A similar [^{3}H]-thymidine incorporation assay was used to quantify proliferation of the 12 colorectal adenocarcinoma cell lines listed in Table 3. Cells were plated at a density of 10,000 cells/well in a 96-well plate and allowed to attach for 24 h in growth medium containing 10% FBS. The cells were then shifted to serum-free medium for 24 h. Subsequently, repifermin (0.01–1000 ng/ml) was added to the cultures. After 22 h, [^{3}H]-thymidine (1 μCi/well, specific activity 5–25 Ci/mmol) was added to the wells, and the cultures were incubated for an additional 2 h. The cells were then harvested, and the incorporated [^{3}H]-thymidine was measured with a beta scintillation counter. To evaluate the effect of repifermin on tumor growth under optimal conditions, this assay was also conducted using complete (10% FBS) medium throughout.

Animals

For the in vivo studies, female athymic NCR-nu nude mice were obtained from the National Cancer Institute, Animal Production Area, Frederick Cancer Research and Development Center (Frederick, Md.). The mice were maintained according to National

Research Council guidelines [11] and in conformance with relevant federal, state, and local regulations. Animal use protocols were reviewed by the Human Genome Sciences' Institutional Animal Care and Use Committee (IACUC). The mice were housed under clean room conditions in sterile cages (five animals per cage) with bedding and water, and food provided ad libitum. The mean weight of the mice at initiation of the in vivo studies was 20 g. Animals were inoculated with tumor cell preparations in the right flank. The tumor-bearing control group were injected intravenously (i.v.) with sterile saline (100 μl) on the same dosing schedule as the animals receiving repifermin. Tumor size was determined two or three times per week by measuring the tumor along two axes using calipers.

In vivo human tumor growth

The first experiment examined the in vivo effect of systemic administration of a single cycle of repifermin on the growth of FaDu cells, a fast-growing, $KGFR^+$ human pharyngeal squamous cell carcinoma line. Female athymic nude mice (mean weight 20 g) were ear-tagged, weighed, and randomly assigned to the following four groups of ten animals each: (1) no treatment; (2) placebo control; (3) 1 mg/kg i.v. repifermin; and (4) 5 mg/kg i.v. repifermin. Immediately following subcutaneous (s.c.) inoculation with 6×10^6 FaDu cells (day 0), mice in the treatment groups were injected with repifermin into the tail vein. Repifermin injections were repeated for a total of four consecutive days (days 0, 1, 2 and 3). Mice were weighed weekly, and tumor size was measured twice a week in a blinded fashion. The duration of the study was 22 days.

The procedure in the second study was similar to that described for the first experiment. Female athymic nude mice (mean weight 20 g, ten per group) were injected s.c. with 2×10^6 Detroit 562, CAL 27, or Caco-2 cells. Following $KGFR^+$ tumor cell injection, a single cycle of repifermin (1 mg/kg) was injected i.v. on days 0, 1 and 2. The duration of the study was 42 days.

In the third study, female athymic nude mice (mean weight 20 g, five per group) were injected s.c. with the following tumor cell types: A-253 (0.5×10^6 cells), FaDu (0.5×10^6 and 1×10^6 cells), Detroit 562 (0.5×10^6 and 2×10^6 cells), or SCC-25 (2×10^6 cells). Following tumor cell injection, repifermin (1 mg/kg) was injected i.v. on days 0–2, 7–9, and 14–16, that is three cycles of three injections. The duration of the study was 23 to 25 days.

In the first three in vivo studies, one or more cycles of repifermin were delivered by the i.v. route. In the fourth set of in vivo experiments, summarized in Table 3, repifermin was injected daily at a dose of 0.2 or 2 mg/kg intraperitoneally (i.p.) for 14 consecutive days, and tumor size measured at the end of that time.

Statistical methods

The mean and standard error of the mean were calculated for each group. A repeat measure analysis of variance (ANOVA) was used to assess the differences between repifermin treatment and placebo (control) groups in the corresponding low or high tumor inoculation conditions. If the model was significant at the 5% level, a post hoc comparison was performed between the active treatment groups and the control group, using Dunnett's multiple comparison procedure.

Results

KGFR (FGFR2iiib) expression in human tumor cell lines

Table 1 summarizes molecular, in vitro and in vivo data from studies with six human carcinomas of the oral cavity and alimentary tract. The KGFR expression level for each cell line was calculated as the ratio of the signal produced by the tumor cells to the signal from normal human primary keratinocytes. The message level for keratinocytes was assigned a value of 1. The highest level of KGFR message expression from the tumors listed in Table 1 was observed in Detroit 562 carcinoma cells, with an eightfold increase in KGFR expression over human keratinocytes. Three other cell lines (Caco-2, A-253 and SCC-25) also expressed KGFR levels above that of primary keratinocytes. Two cell lines (CAL 27 and FaDu) had detectable message for KGFR but expression levels below that of human keratinocytes. Three other human tumor cell lines not shown in Table 1 were evaluated for KGFR expression with the relative expression levels 0.9 (SCC-15), 1.5 (HT-29) and 8.5 (WiDr) but were not evaluated in vivo, although in vitro testing showed no repifermin-mediated proliferation. One human tumor cell line, HEp-2, expressed no KGF receptor and, not unexpectedly, failed to respond to repifermin in vitro.

Table 2 lists human carcinoma cell lines not originally associated with the gastrointestinal (GI) tract. These include carcinomas of the ovary (NIH:OVCAR-3, SK-OV 3, PA-1), bladder (SCaBER), skin (A 431), lung (SW 900), breast (MDA-MB-231), and cervix (SiHa). Three of the eight lines (NIH:OVCAR-3, SCaBER, and A 431) expressed relatively high levels of KGFR message. The other five cell lines (SW 900, SK-OV 3, PA-1, MDA-MB-231, and SiHa) possessed little or no message for the KGF receptor.

Table 3 lists a set of human colorectal adenocarcinoma cell lines. For these tumor cell lines, KGFR message levels were expressed as a fraction of 18S ribosomal RNA. Of the 12 cell lines tested, 4 (LS 174T, SW1417, SW48, and SW480) appeared to contain 100–1000 copies per cell of KGFR message based on quantitation expressed as a fraction of 18S ribosomal RNA ($1–7\times10^{-5}$). Five cell lines (COLO 201, SW403, DLD-1, LoVo, and SW837) had an expression fraction of $1–7\times10^{-6}$, indicating approximately 10–100 copies per cell. Three cell lines (SW620, SW948, and COLO 320DM) had virtually no KGFR expression. For purposes of comparison, IEC-6 cells derived from normal rat intestinal epithelium had an expression fraction of approximately 3×10^{-8} (data not shown).

In vitro effect of repifermin on human tumor cell proliferation

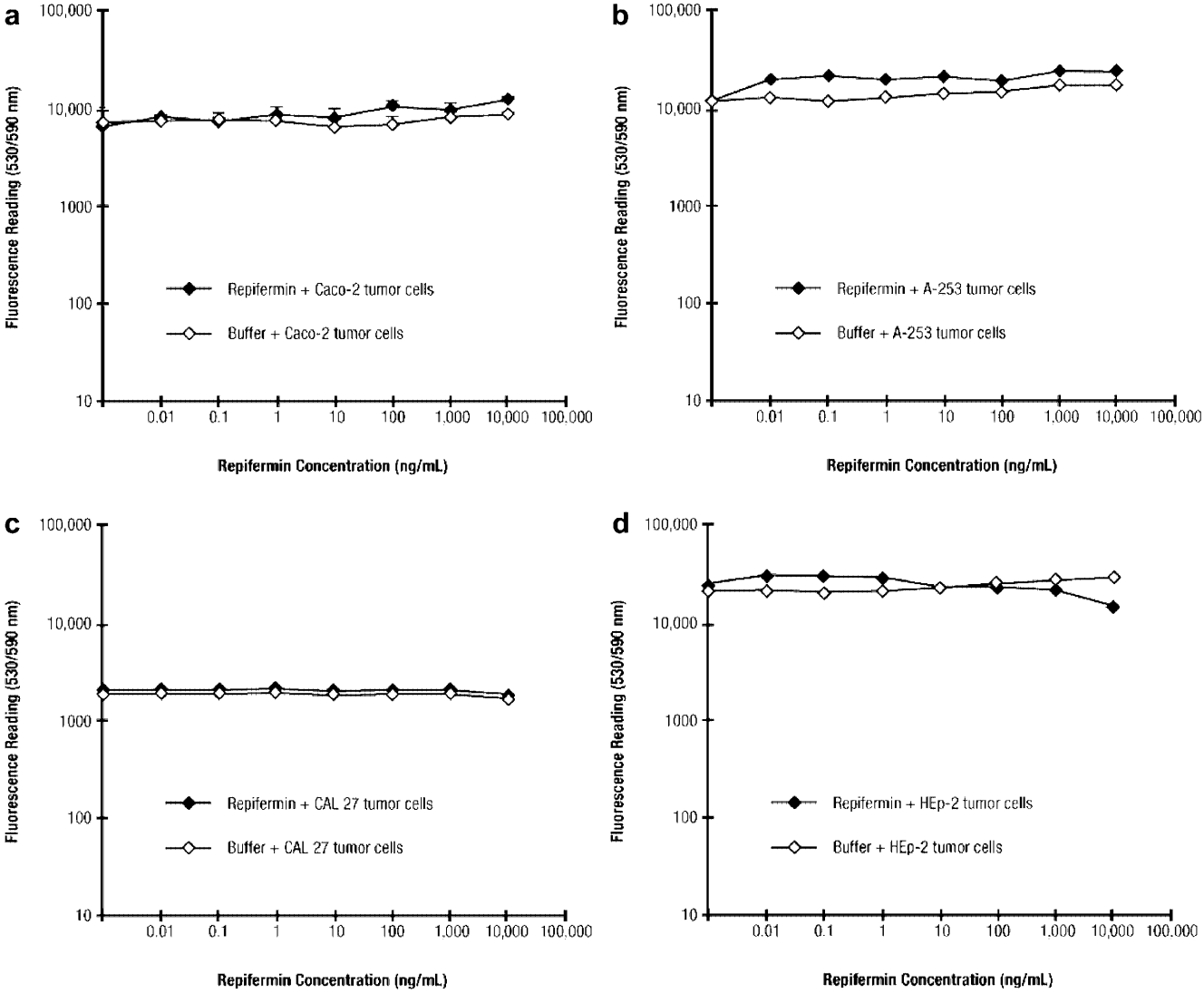
The alamarBlue proliferation assay described in Materials and methods was used to evaluate the in vitro proliferative effects of repifermin (0.01–10,000 ng/ml) on the human carcinoma cell lines listed in Tables 1 and 2. Routinely, repifermin, at a concentration of 1 ng/ml induced a significant two- to threefold increase in primary human keratinocyte proliferation (data not shown). However, at concentrations ranging from 0.01 ng/ml to 10 μg/ml, repifermin failed to significantly elevate proliferation in any of the tumor cell lines listed

in Tables 1 and 2. The full dose-response curve, shown in Fig. 1, is for a representative group of tumor cell lines covering the range of KGFR message expression, that is 7.5 times (Caco-2), 3.5 times (A-253) and 0.9 times (CAL 27) that of human keratinocytes, and no expression of KGFR (HEp-2). No repifermin-mediated proliferation was observed at repifermin concentrations in the range 0.01–10,000 ng/ml in the selected panel of tumor cell lines incubated in 0.1% FBS. The lack of repifermin-associated proliferative activity was confirmed using the [^{3}H]-thymidine incorporation assay with the same series of Caco-2, A-253, CAL 27, and HEp-2 cell lines (Fig. 2).

The human tumor cell lines listed in Table 1 were also grown in medium containing 10% FBS to examine the in vitro effects of repifermin on tumor cells grown under optimal conditions. As in the studies with 0.1% FBS, no significant repifermin-related change in tumor cell proliferation rate was observed using the high 10% FBS concentration in the medium. However, the overall rate of tumor cell proliferation was at least threefold higher in medium with 10% FBS versus that in medium with 0.1% FBS (data not shown).

The human colorectal adenocarcinoma cell lines listed in Table 3 were evaluated in the [^{3}H]-thymidine incorporation assay. All 12 adenocarcinoma cell lines failed to show a response to repifermin (0.01–1000 ng/ml) under serum-free conditions. When the assay was conducted using 5% FBS, 11 of the 12 cell lines continued to exhibit no response to repifermin. The sole exception was the LoVo cell line, which exhibited a statistically significant increase in [^{3}H]-thymidine incorporation at the 10 and 100 ng/ml concentrations (Fig. 3). However, the increases observed were less than

Fig. 1A–D. In vitro effect of repifermin on proliferation of human carcinoma cell lines as measured by alamarBlue staining. Results show the proliferative effects of repifermin (0.01–10,000 ng/ml) on Caco-2 (**A**), A-253 (**B**), CAL 27 (**C**), and HEp-2 (**D**) human carcinoma cell lines. Tumor cells were incubated in medium containing 0.1% FBS for 5 days, then incubated with 10% alamarBlue for 4 to 6 h and read in a fluorescence plate reader. For additional details see "In vitro proliferation assays" in "Materials and methods"



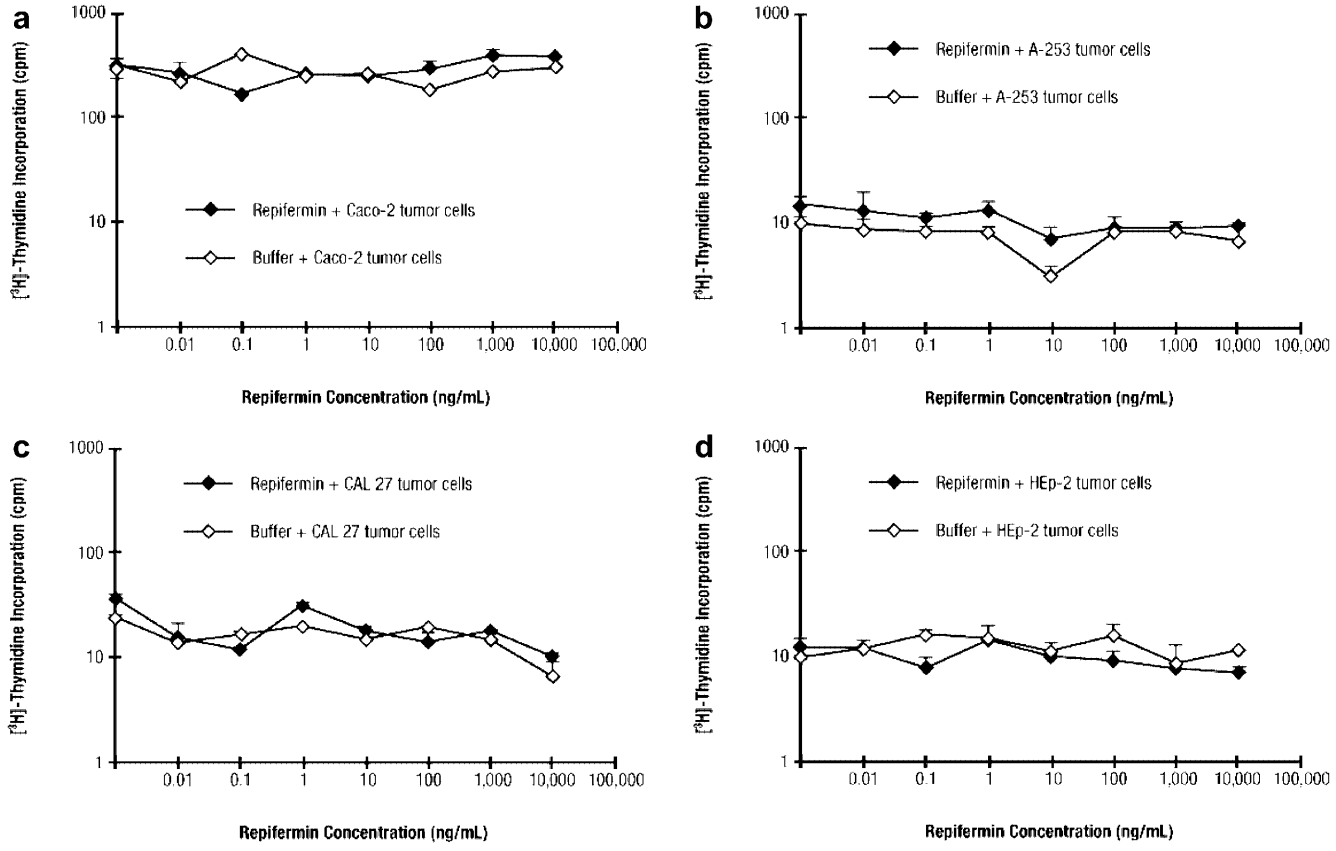


Fig. 2A–D. In vitro effect of repifermin on proliferation of human carcinoma cell lines as measured by [^{3}H]-thymidine incorporation. Results show the proliferative effects of repifermin (0.01–10,000 ng/ml) on Caco-2 (**A**), A-253 (**B**), CAL 27 (**C**) and HEp-2 (**D**) human carcinoma cell lines. Tumor cells were incubated in medium with 10% FBS for 1 day, then incubated with medium containing 0.1% FBS. The cells were pulsed 24 h later with 1 μCi of [^{3}H]-thymidine for 5 h and counted in a beta scintillation counter. For additional details see "In vitro proliferation assays" in "Materials and methods"

50% over the control value (1780 vs 1200 fmol/well) and were not detectable under serum-free conditions. Based on these considerations, and the lack of a dose responsive effect, the response of the LoVo cell line was deemed to be similar to that of the other 11 adenocarcinoma cell lines tested. Thus, in all cases repifermin activity was not sufficient to produce a biologically relevant proliferative response.

In vivo effect of repifermin on growth of human tumors in athymic nude mice

In addition to testing repifermin in vitro for its effects on tumor cell proliferation, repifermin was also tested in a number of in vivo studies. Over the course of a 22-day study with FaDu tumor cells, the implanted tumor grew to a size of approximately 160 mm^2. No tumor growth-promoting activity was found when four consecutive i.v. injections of repifermin (1 or 5 mg/kg) were given to athymic nude mice on days 0–3. There were no statistically significant differences in tumor size between untreated, placebo-injected, and repifermin-treated mice (Fig. 4). Also, no statistically significant differences in body weight change were observed between these groups (data not shown).

In a second study, no tumor growth-promoting activity was found when three consecutive i.v. injections of repifermin (1 mg/kg) were given to groups of athymic nude mice inoculated with Detroit 562, CAL 27, or Caco-2 cells (Fig. 5). There were no statistically significant differences in tumor size between placebo-injected and repifermin-injected mice in any of the three tumors tested. This study indicated no repifermin-mediated stimulatory effects on tumors with widely differing patterns of growth and receptor expression. Detroit 562 cells exhibited high KGFR expression (8.2 times that of primary keratinocytes) and a rapid growth rate (50 mm^2 in 18 days), while CAL 27 had a low KGFR expression (0.9 times) and a moderate growth rate (20 mm^2 in 18 days). The Caco-2 cell line, with its high KGFR expression level (7.5 times) and very low rate of tumor growth (15 mm^2 in 42 days), had a biological profile different from that of both Detroit 562 and CAL 27 cells. With the Caco-2 cells, by day 42, three of the ten animals in each placebo-injected and repifermin-treated group had no detectable tumor. Only animals showing actual tumor growth were included in the calculation of average tumor size. In all cases, no significant differences were seen between placebo-injected and repifermin-treated mice as measured by tumor take or tumor growth rate.

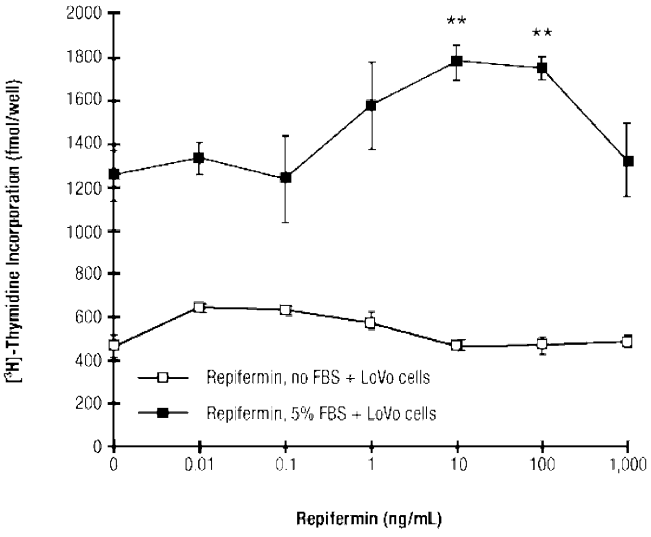


Fig. 3. In vitro effect of repifermin on proliferation of LoVo human carcinoma cells. Results show the proliferative effects of repifermin (0.01–1000 ng/ml) on LoVo cells incubated in medium in the absence or presence of 5% FBS. Tumor cells were pulsed with 1 μCi of [^{3}H]-thymidine for 2 h and counted in a beta scintillation counter. Data points represent the means ± SEM (**$P < 0.01$ repifermin-treated vs vehicle-treated control). For additional details see "In vitro proliferation assays" in "Materials and methods"

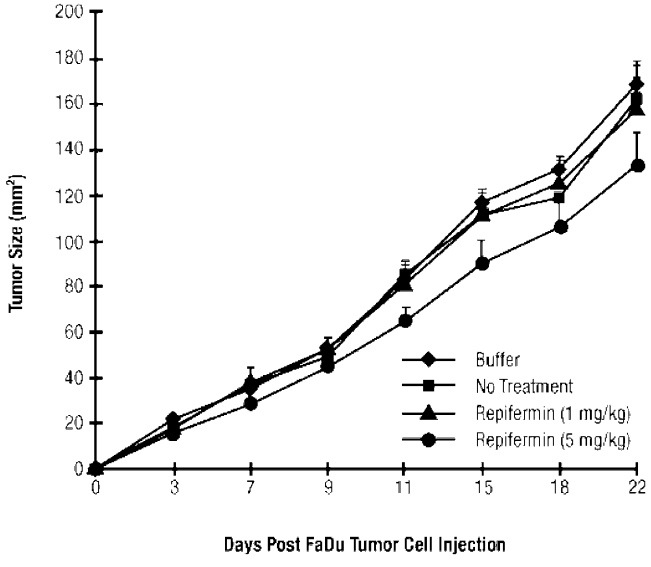


Fig. 4. In vivo effect of repifermin on FaDu tumor growth. FaDu tumor cells (6×10^6) were injected s.c. into nude mice ($n = 10$) on day 0. A single cycle of repifermin (1 or 5 mg/kg, i.v.) was administered on days 0, 1, 2 and 3. Tumor sizes were measured along two axes with calipers approximately twice weekly through day 22, and expressed as means ± SEM (mm^2). Note that the *x*-axis shows the actual days of measurement, and that the scale is not linear. For additional details see "In vivo tumor growth" in "Materials and methods"

In the third study, no tumor growth-promoting activity was found when three weekly cycles of three i.v. injections of repifermin (1 mg/kg) were administered to athymic nude mice inoculated with A-253, FaDu, Detroit 562, or SCC-25 tumor cells (Fig. 6).

A-253 tumor cells were injected s.c. at a cell concentration of 0.5×10^6 cells/mouse. Palpable tumors were present by day 7 and increased in size throughout the experiment. By day 25, the mean tumor size was 54 mm^2 in the placebo group and 49 mm^2 in the repifermin-treated group. Repifermin (1 mg/kg, i.v.) did not significantly alter the A-253 tumor growth pattern, as shown in Fig. 6A.

FaDu cells were inoculated at concentrations of 0.5×10^6 and 1×10^6 cells/mouse. Tumors were palpable by day 9 and increased in size throughout the experiment. By day 23, the mean tumor sizes in the groups receiving the low-dose tumor cell concentration were 66 mm^2 for the placebo group, and 50 mm^2 for the repifermin-treated group. The mean tumor sizes in the high-dose tumor cell groups were 84 mm^2 for the placebo group and 50 mm^2 for the repifermin-treated group. Repifermin (1 mg/kg, i.v.) did not significantly alter the FaDu growth pattern for either the low-dose or high-dose tumor cell groups (Fig. 6B).

Detroit 562 tumor cells were inoculated at concentrations of 0.5×10^6 and 2×10^6 cells/mouse. Tumors were palpable by day 9 and increased in size throughout the experiment. By day 25, the mean tumor sizes in the low-dose tumor cell groups were 34 mm^2 in the placebo group and 30 mm^2 in the repifermin-treated group. The mean tumor sizes in the high-dose tumor inoculum groups were 51 mm^2 in the placebo group and 46 mm^2 in the repifermin-treated group. Repifermin (1 mg/kg, i.v.) did not significantly alter the Detroit 562 growth pattern in the low-dose tumor cell group. However, in the high-dose tumor inoculation group, repifermin caused a slight inhibition of the growth pattern (Fig. 6C).

SCC-25 cells were inoculated at a concentration of 2×10^6 cells/mouse. In contrast to the other three cell lines in this study, SCC-25 tumor cells grew poorly in vivo. Tumors were not palpable until day 14. SCC-25 tumor sizes in the repifermin-treated group varied from 18 mm^2 on day 4 to 11 mm^2 on day 23, whereas the tumor size in the placebo-treated group was approximately 8 mm^2 throughout the experiment. Although these differences in tumor size were statistically significant, the differences were of a very small scale. With the repifermin-treated tumor actually decreasing in size from day 4 to day 23, there was clearly no repifermin-mediated time-dependent increase in the size of the tumor. Therefore, repifermin was considered to be negative for in vivo growth enhancement off the SCC-25 tumor, similar to its effect on the other implanted human carcinomas (Fig 6D).

In the above three in vivo studies, all tumor cell lines were originally derived from tissues associated with the oral cavity or alimentary tract. This is a relevant tissue source supporting current clinical trials in mucositis and ulcerative colitis, as well as planned studies in colorectal and head and neck cancer. However, other human carcinomas, not of alimentary tract origin, were also

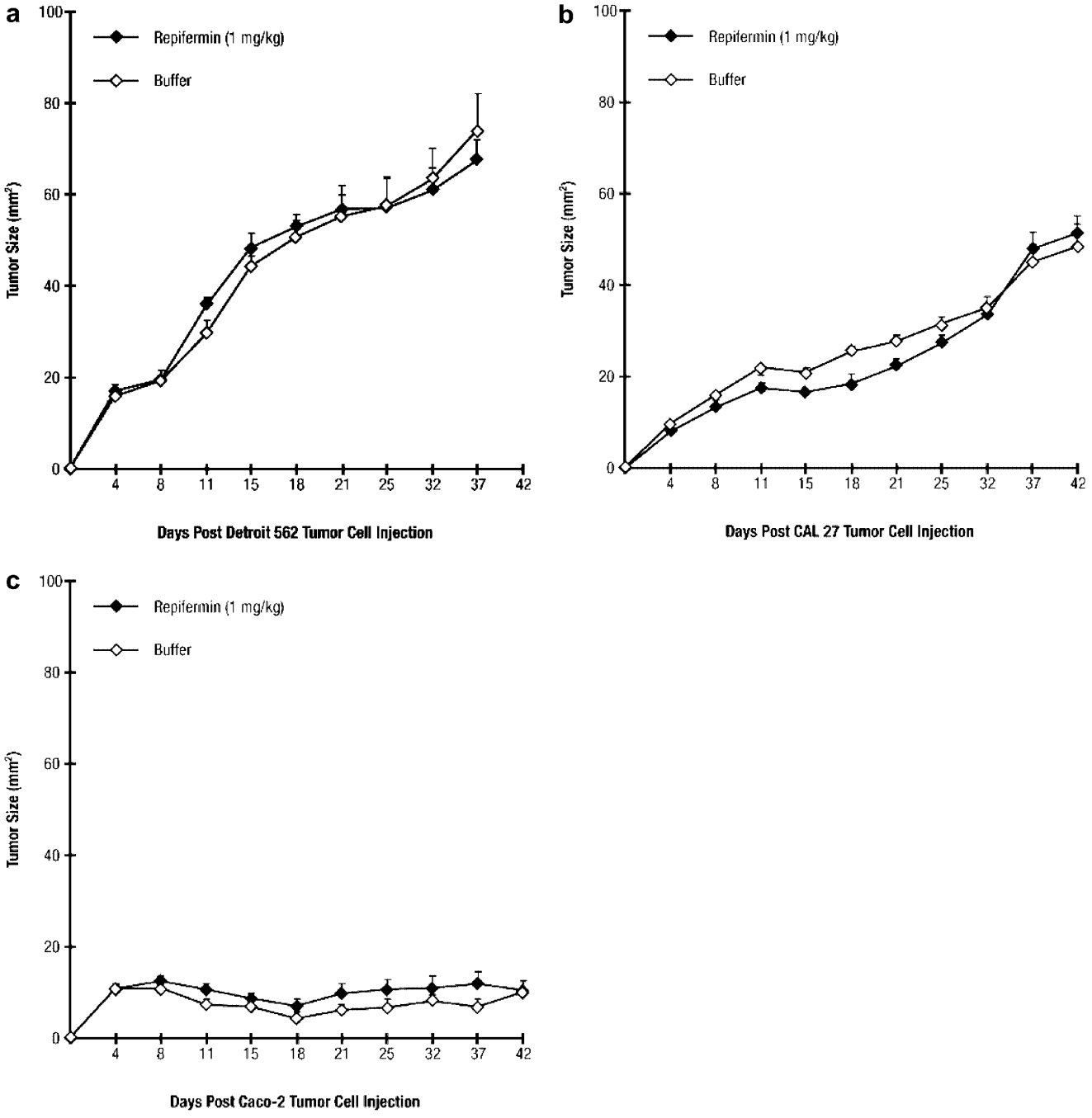


Fig. 5A–C. In vivo effect of repifermin on human carcinomas with varying growth rates and KGFR expression levels. Detroit 562 (**A**), CAL 27 (**B**) and Caco-2 (**C**) tumor cells (2×10^6) were injected s.c. into nude mice ($n=10$) on day 0. A single cycle of repifermin (1 mg/kg, i.v.) was administered on days 0, 1, 2 and 3. Tumor sizes were measured along two axes with calipers approximately twice weekly through day 42, and expressed as means ± SEM (mm^2). Note that the *x*-axis shows the actual days of measurement, and that the scale is not linear for either axis. For additional details see "In vivo tumor growth" in "Materials and methods"

tested for their in vivo response to repifermin. In a fourth study, summarized in Table 2, eight different human carcinomas, originating in the ovaries (NIH:OVCAR-3, SK-OV 3, and PA-1), bladder (SCaBER), epidermis (A 431), lung (SW 900), breast (MDA-MB-231), and cervix (SiHa), were injected s.c. into the flank of nude mice. Repifermin, administered i.p. daily at a dose of 0.2 or 2 mg/kg for 2 weeks, failed to alter the growth pattern of the implanted tumors (data not shown).

Discussion

Because repifermin is an epithelial growth factor intended for systemic administration to cancer patients who may have just been treated for removal of a

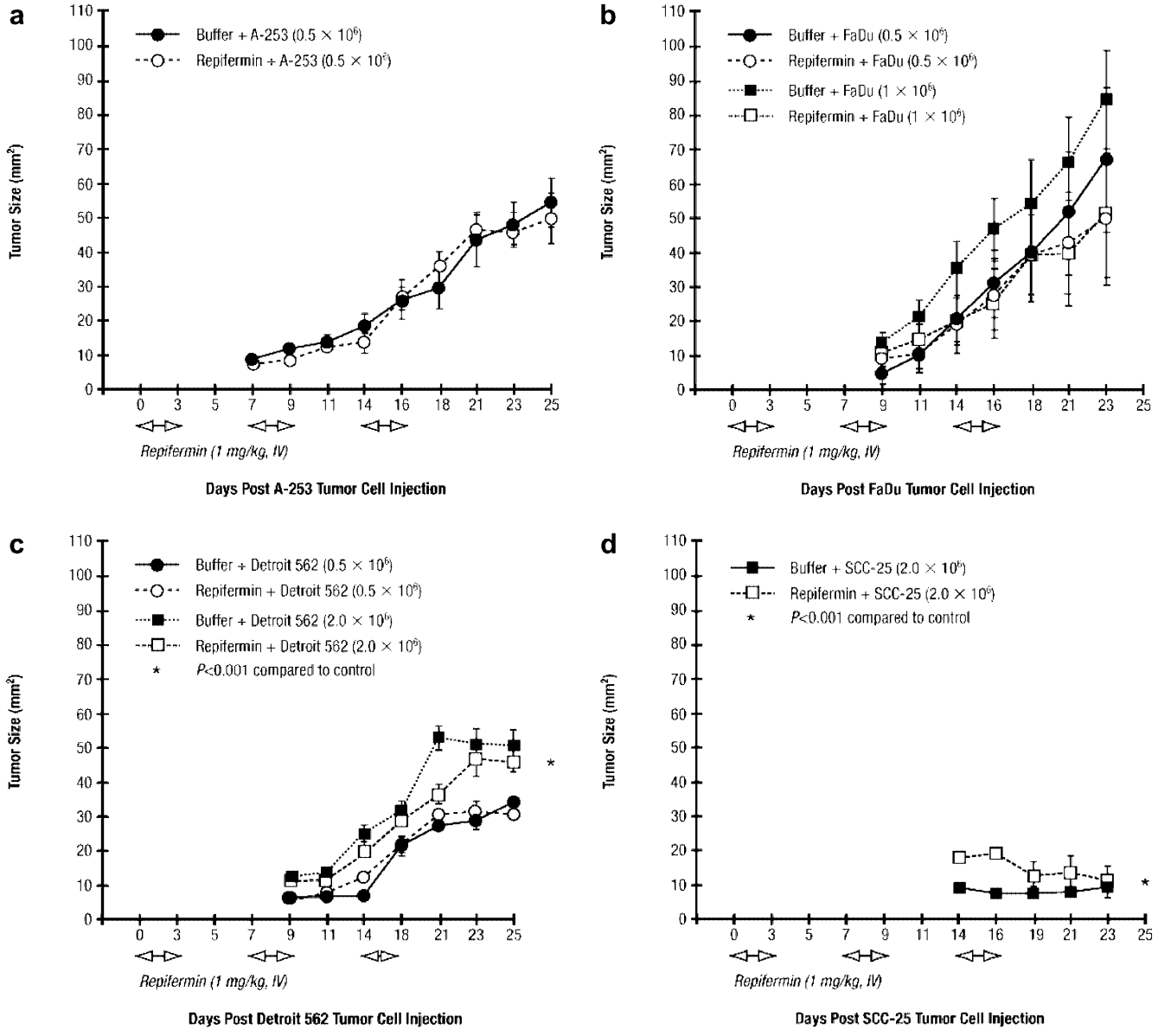


Fig. 6A–D. In vivo effect of three cycles of repifermin treatment on growth of human carcinomas. A-253 (**A**), FaDu (**B**), Detroit 562 (**C**), and SCC-25 (**D**) tumor cells were injected s.c. into nude mice ($n = 5$) on day 0. Following tumor cell injection, repifermin (1 mg/kg, i.v.) was administered on days 0–2, 7–9, and 14–16 (a total of three cycles of three injections). Tumor sizes were measured along two axes with calipers, two to three times weekly until the end of the experiment (day 23 or 25), and expressed as means ± SEM (mm^2). $*P < 0.001$, repifermin-treated vs vehicle-treated control. Note that the *x*-axis shows the actual days of measurement, and that the scale is not linear. For additional details see "In vivo tumor growth" in "Materials and methods"

$KGFR^+$ carcinoma, it was important to establish that repifermin did not induce tumor cell proliferation. The preclinical studies reported here were designed to determine whether repifermin stimulated in vitro or in vivo tumor growth and, by extension, would put patients at risk for developing cancer if used in the clinic. A panel of 30 different tumor cell lines was selected, having in common their human origin and carcinoma cell type. However, the tumor cell panel was compiled to represent tumors from diverse tissue sources, with a range of KGFR expression levels and varying growth rates. Because of the interest in treating cancer patients with oral mucositis, the six carcinomas in Table 1 were predominantly derived from tissues of the oral cavity and alimentary tract. Table 2 shows eight carcinomas of more diverse origin including ovary, breast and lung. The 12 tumors shown in Table 3 are colorectal adenocarcinomas, chosen because of the interest in treating cancer-prone ulcerative colitis patients as well as the GI mucositis associated with colorectal cancer patients recovering from chemotherapy and radiation. As measured by quantitative PCR, the carcinomas also showed a spectrum of KGFR expression levels, ranging from the 11-fold increase over primary human keratinocytes in the NIH:OVCAR-3 ovarian tumor cell line to the

absence of KGFR expression in the MDA-MB-231 breast adenocarcinoma.

The in vitro studies were conducted under optimal (5–10% FBS) and suboptimal (0.1% or no FBS) growth conditions. None of the 18 carcinoma cell lines tested in the first two series of in vitro experiments exhibited any positive or negative proliferative response to repifermin (0.01–10,000 ng/ml) as assessed by the alamarBlue or [^{3}H]-thymidine assay at 0.1 and 10% FBS. In Table 3, the 12 adenocarcinoma cell lines were tested in the absence or presence of 5% FBS and assessed by [^{3}H]-thymidine incorporation. None could be identified that exhibited a substantial proliferative response to repifermin at concentrations in the range 0.01–1000 ng/ml.

In addition to the in vitro experiments, a series of four in vivo studies were conducted, in which human carcinomas were injected into the flank of nude mice and tumor growth over time following a single cycle or multiple cycles of repifermin was measured. A single cycle of three or four i.v. repifermin injections at a dose of 1 or 5 mg/kg failed to enhance tumor growth, whether the tumor was a low KGFR expresser with a rapid growth rate (FaDu, Fig. 4), a high KGFR expresser with a rapid growth rate (Detroit 562, Fig. 5A), a low KGFR expresser with a moderate growth rate (CAL 27, Fig. 5B), or a high KGFR expresser with a very low growth rate (Caco-2, Fig. 5C).

In a third set of in vivo experiments, repifermin (1 mg/kg) was administered for three cycles of three i.v. injections per week. Under these conditions, the growth of the A-253 (Fig. 6A), FaDu (Fig. 6B) and Detroit 562 (Fig. 6C) tumors was not enhanced by injection of repifermin. The SCC-25 tumors (Fig. 6D), like the Caco-2 tumors in Fig. 5C, had a very slow growth rate that was not accelerated by repifermin treatment.

A fourth set of in vivo studies was conducted on carcinomas from a variety of tissues (Table 2). When given daily for 14 days via the i.p. route, repifermin (0.2 or 2 mg/kg) failed to alter the growth pattern of any tumor tested. These studies indicate that, despite their expression of the FGFR2iiib receptor, in vivo growth of ten human tumor cell lines (Detroit 562, Caco-2, A-253, SCC-25, CAL 27, FaDu, NIH:OVCAR-3, SCaBER, A 431 and SW 900) was not enhanced by repifermin administration.

There is some precedent for growth factors having a selective proliferative effect on normal, but not neoplastic tissue. Ning et al. found that KGF-1, also known as FGF-7, had no proliferative effect on seven KGFR$^+$ tumor cell lines when tested over a 48-h incubation period [20]. When the in vitro exposure was lengthened to 7 or more days, there was a modest but significant KGF-1-mediated increase in proliferation of the Detroit 562, FaDu, SCC-25 and SCC-9 tumor cell lines. However, that minimal in vitro activity did not translate into an enhancement of tumor growth in vivo [20]. In our hands, repifermin stimulated neither in vitro nor in vivo growth of Detroit 562, FaDu and SCC-25 tumors (Table 1). In this regard, it is different from epithelial growth factor (EGF), which has been shown to stimulate growth of selected tumors [3].

Okunieff et al. have demonstrated that KGF-2 not only fails to enhance in vivo tumor growth but actually significantly reduces it, although they had no concrete explanation for the observed antitumor effect against KHT sarcomas [21]. In our in vivo studies, repifermin injected i.v. did significantly inhibit the growth of a Detroit 562 tumor (Fig. 6C), but like Okunieff et al., we do not understand the mechanism of action behind that inhibition, although Okunieff et al. did state that ''short exposures to KGFs do increase tumor cell apoptosis'' [21]. Ding et al., evaluating the in vivo activity of the FGF family, found that i.v. infusion of acidic or basic FGF (FGF1 and 2) did not stimulate growth of three murine tumors [4], again demonstrating that growth factors that stimulate normal cell proliferation do not necessarily affect tumors cells in the same way.

In the clinical setting it is not enough for a growth factor to lack tumor proliferative activity. It must also not negatively impact the chemo- or radiosensitivity of the tumor [24]. In the three aforementioned studies, pretreatment with KGF-1 [20, 21], KGF-2 [21], FGF-1 [4] or FGF-2 [4, 21] did not result in radioprotection to the irradiated tumor. In studies with another growth factor, EGF, several groups have found that this protein actually makes the tumor more, not less, sensitive to the effects of irradiation [16] and chemotherapeutic agents [15, 19]. In our laboratory, studies to evaluate the effect of repifermin on irradiated tumors are underway. However, initial in vivo studies in nude mice have already demonstrated that repifermin pretreatment has no chemoprotective effects on KGFR$^+$ human adenocarcinomas treated with 5-FU (FASEB 2002 abstract, ''Repifermin does not enhance human colorectal adenocarcinoma growth or diminish 5-FU anti-tumor activity in mice'').

Although there are numerous instances of growth factors inducing proliferation of normal but not tumor cells, the mechanism behind this selective activity is not clearly understood. Ning et al. [20] speculate that the lack of response by tumor (but not normal) cells may be due to a difference in the affinity of the KGF receptor or low receptor protein expression despite measurable levels of mRNA. They also hypothesize that normal and tumor cells may have different regulatory pathways associated with their respective KGF receptors. Since neoplasia is, by definition, characterized by unregulated proliferation, exposing tumor cells to a growth factor such as repifermin may have no more significant effect than adding a drop of water to a pond. In normal tissues, the KGF receptor is a tyrosine kinase activated by the process of binding to its ligand [17]. The activated phosphorylated receptor, in turn, triggers phosphorylation of phospholipase C gamma (PLC gamma), a common second messenger protein involved in proliferation and differentiation [22]. Interestingly, when normal salivary gland mRNA from repifermin-injected mice is incubated with commercially available murine gene

arrays, one of the messages that is dramatically upregulated is PLC gamma (unpublished data). Although no tumor mRNA tissue was tested in this study, it is possible that the signal transduction pathways in normal and tumor cells are differentially affected by repifermin. In our most recent gene array experiments with activated salivary gland target tissue, another signal transduction messenger, MAPK3, was upregulated. There are efforts underway to accumulate mRNA from a variety of tumor cell lines and evaluate them in similar arrays.

Even though the explanation for the failure of repifermin to augment the proliferation of $KGFR^+$ tumors is not clear, that inactivity was reproducibly demonstrated in a number of in vitro and in vivo experiments using epithelial-like tumors from a variety of tissues with a range of receptor expression and rate of proliferation. The lack of repifermin-mediated proliferative effects on these $KGFR^+$ human carcinomas both in vitro and in vivo suggests that the drug may be developed further for use in indications such as chemotherapy- or radiation therapy-induced mucositis, inflammatory bowel disease, or other disorders involving damage to epithelial tissues.

Acknowledgements We thank Julian Kaye and Linda Roux for their expertise in helping compile the manuscript, as well as Zebedee Okoye, Gemma Santiago, Kathleen Windle, and Eling Wong for their technical assistance. For purposes of disclosure, note that all authors are employees of Human Genome Sciences, Inc.

References

1. Ahmed SA, Gogal RM Jr, Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assay. J Immunol Methods 170:211
2. Bansal GS, Cox HC, Marsh S, Gomm JJ, Yiangou C, Luqmani Y, Coombes RC, Johnston CL (1997) Expression of keratinocyte growth factor and its receptor in human breast cancer. Br J Cancer 75:1567
3. Carpenter G, Cohen S (1990) Epidermal growth factor. J Biol Chem 265:7709
4. Ding I, Huang K, Snyder M, Cook J, Zhang L, Wersto N, Okunieff P (1996) Tumor growth and tumor radiosensitivity in mice given myeloprotective doses of fibroblast growth factors. J Natl Cancer Inst 88:1399
5. Drugan CS, Stone A, Game SM, Prime SS (1997) The mitogenic effect of KGF and the expression of its cell surface receptor on cultured normal and malignant human oral keratinocytes and on contiguous fibroblasts. J Oral Pathol Med 26:327
6. Emoto H, Tagashira S, Mattei M-G, Yamasaki M, Hashimoto G, Katsumata T, Negoro T, Nakatsuka M, Birnbaum D, Coulier F, Nobuyuki I (1997) Structure and expression of human fibroblast growth factor-10. J Biol Chem 272:23191
7. Farrell C, Bready J, Rex K, Chen J, DiPalma C, Whitcomb K, Yin D, Hill B, Wiemann B, Lacey D (1998) Keratinocyte growth factor protects mice from chemotherapy and radiation-induced gastrointestinal injury and mortality. Cancer Res 58:933
8. Han D, Li F, Holt L, Connolly K, Hubert M, Miceli M, Okoye Z, Santiago G, Windle K, Wong E, Sartor B (2000) Keratinocyte growth factor-2 (FGF-10) promotes healing of experimental small intestinal ulceration in rats. Am J Physiol Gastrointest Liver Physiol 279:G1011
9. Hay R, Caputo J, Chen T, Macy M, McClintock P, Reid Y (1994) Cell lines and hybridomas, 1994 Reference Guide, 8th edn. American Type Culture Collection, Manassas
10. Igarashi M, Finch PW, Aaronson SA (1998) Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J Biol Chem 273:13230
11. Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (1996) Guide for the care and use of laboratory animals. National Academy Press, Washington, DC
12. Ishiwata T, Friess H, Büchler MW, Lopez ME, Korc M (1998) Characterization of keratinocyte growth factor and receptor expression in human pancreatic cancer. Am J Pathol 153:213
13. Jimenez PA, Greenwalt D, Mendrick DL, Rampy MA, Su J, Leung KH, Connolly KM (2000) Keratinocyte growth factor-2. In: Narula SK, Coffman R (eds) New cytokines as potential drugs. Birkhäuser Verlag, Basel, p 101
14. Khan W, Shui C, Ning S, Knox S (1997) Enhancement of murine intestinal stem cell survival after irradiation by keratinocyte growth factor. Radiat Res 148:248
15. Kroning R, Jones J, Hom D, Chuang C, Sanga R, Los G, Howell S, Cristen R (1995) Enhancement of drug sensitivity to human malignancies by epidermal growth factor. Br J Cancer 72:615
16. Kwok T, Sutherland R (1989) Enhancement of sensitivity of human squamous carcinoma cells to radiation by epidermal growth factor. J Natl Cancer Inst 81:1020
17. Marchese C, Felici A, Visco V, Lucania G, Makoto I, Picardo M, Frati L, Torrisi M (2001) Fibroblast growth factor 10 induces proliferation and differentiation of human primary cultured keratinocytes. J Invest Dermatol 116:623
18. Miceli M, Hubert M, Santiago G, Yao D, Connolly K (1999) Effect of keratinocyte growth factor-2 on dextran sulfate sodium induced colitis in mice. J Pharm Exp Ther 290:464
19. Murayama Y (1990) Growth-inhibitory effects of epidermal growth factor on human breast cancer and carcinoma of the esophagus transplanted into nude mice. Ann Surg 211:263
20. Ning S, Shui C, Khan WB, Benson W, Lacey DL, Knox SJ (1998) Effects of keratinocyte growth factor on the proliferation and radiation survival of human squamous cell carcinoma cell lines in vitro and in vivo. Int J Radiat Oncol Biol Phys 40:177
21. Okunieff P, Li M, Liu W, Sun J, Fenton B, Zhang L, Ding I (2001) Keratinocyte growth factors radioprotect bowel and bone marrow but not KHT sarcoma. Am J Clin Oncol 24:491
22. Powers C, McLeskey S, Wellstein A (2000) Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7:165
23. Soler P, Wright T, Smith P, Maggi S, Hill D, Ko F, Jimenez P, Robson M (1999) In vivo characterization of keratinocyte growth factor-2 as a potential wound healing agent. Wound Repair Regen 7:172
24. Stone H, McBride W, Coleman C (2002) Modifying normal tissue damage post irradiation. Report of a workshop sponsored by the Radiation Research Program, National Cancer Institute. Radiat Res 157:204