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Experimental radiobiology

Effects of keratinocyte growth factor (palifermin) administration protocols on oral mucositis (mouse) induced by fractionated irradiation

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Abstract

***Background and purpose*: Aim of this study was to assess the impact of the administration protocol of palifermin on amelioration of oral mucositis after fractionated irradiation.**

***Materials and methods*: Mouse tongue ulceration was analysed as the clinically relevant endpoint. Daily fractionated irradiation (5×3 Gy/week, days 0 to +4, +7 to +11, with a weekend gap on days +5 and +6) was followed by graded test doses on day +14, i.e. after a second weekend gap. Palifermin (5 mg/kg) was injected subcutaneously. In the first series of experiments, the effect of three daily injections (days −3, −2 and −1) was compared with a single administration either on day −2 or −1; all animals received a further injection on day +4. In the second series, a single or three injections were given in the weekend gap between fractionated irradiation (days +5 to +6), with an additional administration on day +11. In a final protocol, single weekly injections of palifermin were given either on days −3, +4 and +11, days +4, +11 and +18, or on days −3, +4, +11 and +18.**

***Results*: The ED50 (dose after which ulcer induction is expected in 50% of the mice) to single dose irradiation was 11.5±0.7 Gy. The ED50 for test irradiation after 10×3 Gy was 5.7±1.6 Gy. Palifermin administration before the start of fractionated irradiation and on day +4 increased the ED50 to 10-12 Gy, administration over the first weekend and on day +11 to 11-15 Gy. Administration over three consecutive weekends, starting on day −3 or day +4, increased the ED50 to 13.0±0.1 and 14.9±0.3 Gy. Single weekly KGF administrations over four weekends, including the weekend prior to and after completion of radiotherapy, showed no further increase in ED50.**

***Conclusions*: A single palifermin injection during the weekend gap before or during fractionated irradiation is as effective as three applications. Onset of the palifermin treatment during the first weekend gap between fractionated irradiation is more effective than during the weekend before radiotherapy. The effect of palifermin on oral mucositis can be increased by three weekly injections, while four injections do not yield a further increase in ED50.**

Keywords: Oral mucositis; Fractionated irradiation; Growth factors; Mouse model

Surface epithelia, such as oral mucosa or epidermis, are turnover tissues, where permanent cell loss by mechanical stress at the surface is precisely balanced by continuous proliferation in deeper, germinal epithelial layers, and subsequent differentiation of the produced cells. Impairment of proliferation by irradiation eventually results in a reduction of the cellular supply to the postmitotic cell layers. Cells present at the time of radiation exposure undergo near normal differentiation [13,24]. The ongoing cell loss, which is dependent on the biological turnover rate of the tissue, but largely independent of the radiation dose, continues at an almost normal rate after irradiation [6,12].

The imbalance between proliferation and cell loss after sufficient doses eventually yields complete cellular depletion, which in oral mucosa manifests as focal to confluent ulceration. The clinical response is associated with a significant impairment of the epithelial barrier function against mechanical and chemical stress as well as against adhesion and penetration of microbia.

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family (FGF-7). KGF is almost exclusively synthesized by mesenchymal cells, particularly fibroblasts. The receptor is a tyrosine kinase that is expressed by epithelial cells in a variety of tissues [31].

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The response of epithelial tissues to KGF includes stimulation of cell production as well as modification of migration and differentiation processes, i.e. cellular functions.

In previous experimental studies, highly significant amelioration of the radiation-induced mucosal response by recombinant human KGF (rHuKGF, palifermin) has been demonstrated for single dose and fractionated irradiation [7,15,16]. However, the growth factor had to be applied before the onset of clinical mucositis; no effect was seen at the time of manifest ulceration [17]. In combination with daily fractionated irradiation over 1 week [16], one injection of KGF before the onset of radiotherapy was as effective as daily administration over 3 days before the first radiation fraction. Similarly, the effect of only one injection at the end of the first treatment week was identical to that of three injections over the first weekend break.

The present investigation was therefore initiated to define the efficacy of only one palifermin injection per weekend compared to repeated injections over three consecutive days, and the impact of the day when the single injection was given. For this, a combination of varying administration protocols over the weekend before radiotherapy started or over the first weekend break were compared. These were combined to one further injection at the subsequent weekend, i.e. day +4 or day +11, respectively. Moreover, single injections per weekend over three or four consecutive weekends were tested.

In all experiments, the incidence of mucosal ulceration, equivalent to oral mucositis grade 3 RTOG/EORTC or CTCv3 AE, was used as a clinically relevant endpoint for radiation dose–response analyses. Residual tissue tolerance after 2 weeks of fractionated irradiation—without or with palifermin—was determined by graded test doses. The effect of palifermin was then defined by comparison of the resulting dose–effect curves for test irradiation.

Material and methods

Animals and housing

Mice of the inbred C3H/Neu strain, bred in the colony of the Medical Faculty Carl Gustav Carus, were used in all experiments. Previous studies did not reveal any gender-associated differences in the mucosal radiation response [9], and hence both sexes were included. The mice were bred and housed under specified pathogen-free (SPF−) conditions. Conditions of humidity (30–50%) and temperature (21–24 °C) were automatically regulated. A light programme controlled a 12/12-h light dark-rhythm, with lights on from 6 a.m. to 6 p.m.

For housing, size 3 Macrolon®-cages (Tecniplast Pereg GmbH, Waldkraiburg, Germany) with saw-dust bedding (Sniff $^{3}/_{4}$, Altrogge, Lage, Germany) were used. Maximally 10 mice were housed per cage. Standard mouse diet (Altromin 1326, Altrogge) and filtered city tab water from standard perspex drinking bottles were provided ad libitum.

Irradiation technique

For radiation treatment, a combination of two techniques was used: percutaneous irradiation of the entire snout and local top-up treatment of a test area at the lower tongue surface. Set-up and technique for both radiation treatment of the snout and of the lower tongue surface were recently reported in detail [9,16,28].

For *snout irradiation*, no anaesthesia was required. The animals were guided into plastic tubes with an inner diameter of 28 mm. A conical hole in a perspex block at the front end of the tubes served for standardised positioning of the snout. The rear ends of the tubes were closed to prevent withdrawal by the animals. Eight animals were irradiated in parallel. For this, two opposing rows with four tubes each were arranged on a perspex plate. Shielding of the body, behind a plane from the eyes to the throat, was achieved by 6 mm of lead equivalent MCP-96 (HEK Medizintechnik, Lübeck, Germany). The treatment fields defined by this collimation plate encompassed the snouts including the entire tongue.

An 'Isovolt 320/20' X-ray device (Seifert Röntgenwerke, Ahrensburg, Germany) was used for snout irradiation, operated at 200 kV with a tube current of 20 mA. The beam filter of 0.6 mm Cu and 1 mm Al resulted in a dose rate of 1.07 Gy/min at the focus-to-skin distance of 45.5 cm.

The dose rate was regularly checked and found highly stable. This allowed for adjustment of the treatment dose by definition of the time for irradiation. The dose homogeneity between the individual snout treatment fields was ±3%.

Test irradiation was given to a treatment field at the central lower tongue. A DARPAC 150-MC device (Forward Raytech Ltd) was operated at 25 kV with a tube current of 20 mA. The beam filter of 0.3 mm Al resulted in a dose rate of 3.78 Gy/min at the focus-to-skin distance of 15 cm. The dose rate was checked regularly by medical physicists of the radiotherapy department and found constant. Hence, the dose was defined by adjustment of the irradiation time.

Immobilisation of the animals for local irradiation was achieved by intraperitoneal administration of Pentobarbitone sodium (Narcoren®, Rhone Merieux) at a dose of 60 mg/kg. The immobilised mice were placed in the central bore (diameter 25 mm) of a pre-warmed aluminium block (~35 °C) in a supine position. The tongue was gently guided through a hole in the roof of the block (diameter: 3 mm) by means of a forceps. The upper tongue surface was fixed to the outer surface of the block by double adhesive tape. Subsequently, the head was supported by a polystyrene wedge to avoid traction at the base of the tongue.

A $3 \times 3\ mm^2$ window in a 1 mm thick aluminium plate defined the treatment area. The window was positioned centrally over the tongue, shielding base, margins and tip.

Palifermin (keratinocyte growth factor)

Recombinant human KGF, produced in *E. coli*, was provided by AMGEN, Inc., Thousand Oaks, CA 91320-1789, USA. Purification to homogeneity was achieved by conventional chromatography. Endotoxin tests were performed by AMGEN, Inc.

The lyophylisate was dissolved in the reconstitution solution provided by the company, consisting of sterile water and Tween 20, at a concentration of 5.21 mg/ml, and was further diluted in sterile phosphate buffered saline (PBS) to a final concentration of 1 mg/ml.

The solution was freshly prepared at each day of injection. The final concentration rendered injection volumes of 0.1–0.12 ml per mouse for a dose of 5 mg/kg per injection; KGF was administered subcutaneously. Dosage and route of administration was based on previous studies in mice [15–19].

Experimental design

Single dose irradiation alone

In a control experiment, graded single doses, in order to generate a full dose–effect curve, were administered to the lower tongue surface. This experiment was included to define the radiation tolerance of normal, untreated mouse tongue and the time course of the response. Five dose groups, with eight animals each, were used.

Fractionated irradiation

Daily fractionated irradiation was applied with five fractions of 3 Gy per week, given over 2 weeks (days 0 to +4, +7 to +11), with a weekend gap (days +5 to +6). The fractionated treatment was followed by graded local test doses (top-up, TU) after a second weekend gap, i.e. on day +14 (Fig. 1). Test irradiation comprised five dose groups with 10 animals each, to generate a full dose–effect curve. In this control experiment, no palifermin was administered.

Palifermin treatment protocols

The experimental protocols are illustrated in Fig. 1. In all experiments, palifermin was applied subcutaneously at a dose of 5 mg/kg per injection.

The first two experimental arms focused on the influence of the number of injections per weekend, and the timing of these injections on the protective effect of palifermin. In the first series of experiments, the effect of three daily injections at the weekend before the onset of radiotherapy (days −3, −2 and −1) were compared with a single administration either on day −2 or −1; all animals received a further injection on day +4. In the second series, a single or three injections were given in the weekend gap between fractionated irradiation (days +5 to +6), with an additional administration on day +11. In a final protocol, single weekly injections of palifermin were given either on days −3, +4 and +11, days +4, +11 and +18, or on days −3, +4, +11 and +18.

Follow-up, end-point, and statistical analysis

Mucosal ulceration, corresponding to confluent mucositis RTOG/EORTC or CTC AEv3 grade 3, was used as the clinically relevant, quantal endpoint in all studies. Radiation effects in the tongue epithelium were scored daily from the onset of first symptoms until complete re-epithelialisation. For this, the animals were immobilised by ultra-short anaesthesia with Methohexitone (Brevimytal®, Lilly) at a dose of ~40 mg/kg, given intraperitoneally. Scoring was performed under a cold light source.

The frequency of animals responding with ulceration was used for dose–effect analyses. Further parameters to describe the radiation response were latent time, defined as time from test irradiation to first diagnosis of ulcer, and individual ulcer duration.

For all statistical procedures, the Statistical Analysis System, SAS, was used [32]. Probit analyses were used to establish dose–effect relationships, assuming a log-normal distribution (logit analysis). This revealed ED50-values, i.e. doses at which a positive reaction (ulceration) was expected in 50% of the animals treated, and their standard deviation σ. Also, *P*-values for the effect of dose on ulcer induction were calculated, based on the slope of the regression line of the probit curve.

Dose–effect relationships were compared by maximum-likelihood analyses, applying a likelihood-ratio-test on the basis of the logit model, without the assumption of a threshold dose [32].

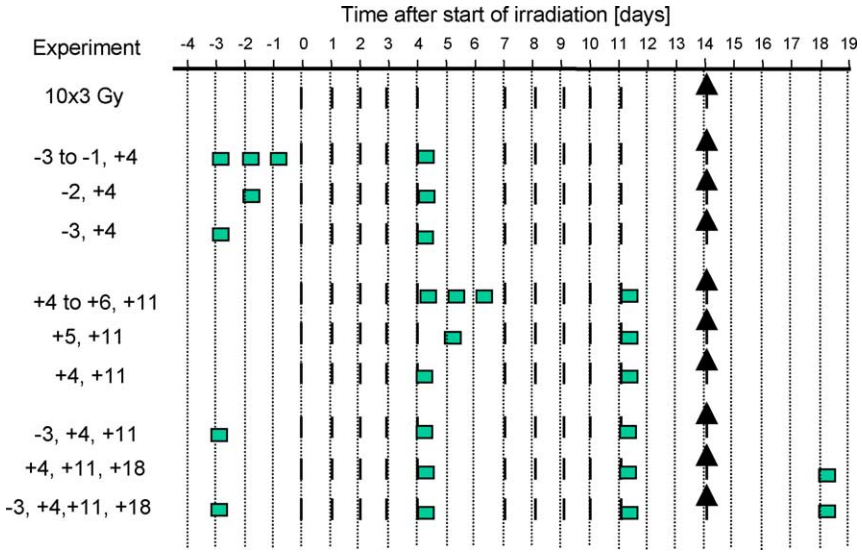

Fig. 1. Experimental protocols. The irradiation protocols in the present study comprised fractionated irradiation with 3 Gy/fraction (bars) followed by test irradiation with graded doses (▲). Palifermin administration intervals are indicated by shaded boxes. The growth factor was administered daily, at a dose of 5 mg/kg per day.

Results

Single dose irradiation

The ED50-value for single dose irradiation alone was 11.5±0.7 Gy. Ulcer incidence was highly dose-dependent ($P=0.006$). Mean latent times were 9.8±2.3 days; the ulcers on average lasted for 4.2±1.2 days (mean±SD). This was in good agreement with previous results [9,15-17,28]. Similar to these previous studies, the treatment was well tolerated and no acute morbidity other than the mucosal response was observed.

Fractionated irradiation

Fractionated snout irradiation with 10×3 Gy/12 days resulted in an ED50 for test irradiation (day +14) of 5.7±1.5 Gy (Table 1). Average latent time (from the day of test irradiation) was 6.2±0.7 days, mean ulcer duration 2.2±0.7 days (mean±SD). Again, the treatment was well tolerated.

Palifermin treatment protocols

The results of the palifermin experiments are summarised in Table 1. In all protocols, a significant increase in isoeffective radiation doses, i.e. ED50-values were observed.

Palifermin administered over the entire weekend prior to the onset of radiotherapy (days −3, −2 and −1), with an additional single injection on day +4, resulted in an ED50 of 12.3±1.8 Gy (P vs. control: 0.0001). One injection on day −2 or −3, plus one injection on day +4, yielded similar ED50-values of 12.2±0.1 Gy ($P=0.001$) and 12.1±0.1 Gy ($P=0.0001$), respectively. Hence, one single injection over the weekend before the first radiation fraction was as effective as three injections, independent of the day when the single administration was given (Fig. 2).

Administration of palifermin in the first weekend gap, i.e. on days +4, +5 and +6, with an additional injection on day +11, resulted in an ED50 of 12.8±1.1 Gy (P vs. control: 0.0001). Two injections, on days +5 and +11 or on days +4 and +11, increased the ED50 to 14.3±1.4 Gy (P vs. 3 inj.: 0.0965) and 14.0±0.1 Gy (P vs. 3 inj.: 0.1242), respectively (Fig. 2). Again, one single injection over the first weekend gap in the fractionated treatments was as effective as three injections, independent of the weekend day at which the single administration was given.

With three injections of palifermin, either on days −3, +4 and +11, or on days +4, +11 and +18, ED50-values of 13.0±0.1 and 14.9±0.3 Gy were observed (Fig. 3). A fourth injection (days −3, +4, +11, +18) did not further change the isoeffective dose, with an ED50 of 13.0±0.1 Gy.

The time course of the mucosal response, assessed by latent times and ulcer durations, was largely independent of

Table 1
Effect of palifermin administration protocol on mouse tongue reactions to fractionated irradiation

Palifermin treatment days	ED50 (Gy)	σ (Gy)	P vs. control
- (control)	5.7	1.5	-
−3 to −1, +4	12.3	1.8	<0.0001
−2, +4	12.2	0.1	0.001
−3, +4	12.1	0.1	<0.0001
+4 to +6, +11	12.8	1.1	<0.0001
+5, +11	14.3	1.4	<0.0001
+4, +11	14.0	0.1	<0.0001
−3, +4, +11	13.0	0.1	<0.0001
+4, +11, +18	14.9	0.3	<0.0001
−3, +4, +11, +18	13.0	0.1	<0.0001

The experimental code refers to Fig. 1. Dose-effect analyses for test irradiation were done by probit analysis. For comparison of dose-effect curves, maximum-likelihood analysis was applied with a likelihood-ratio-test on the basis of the logit model, without the assumption of a threshold dose [32].

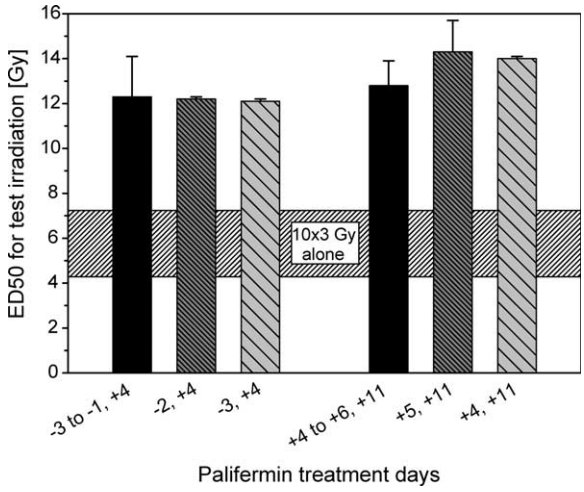

Fig. 2. ED50-values for test irradiation. ED50-values, i.e. doses at which a positive mucosal response was expected in 50% of the animals, and their standard deviation σ (error bars) were calculated by probit analyses. The experimental code given at the abscissa refers to Fig. 1 and gives the days of injection of 5 mg/kg of palifermin. The hatched area indicates ED50±σ for top-up irradiation after 10×3.0 Gy without palifermin.

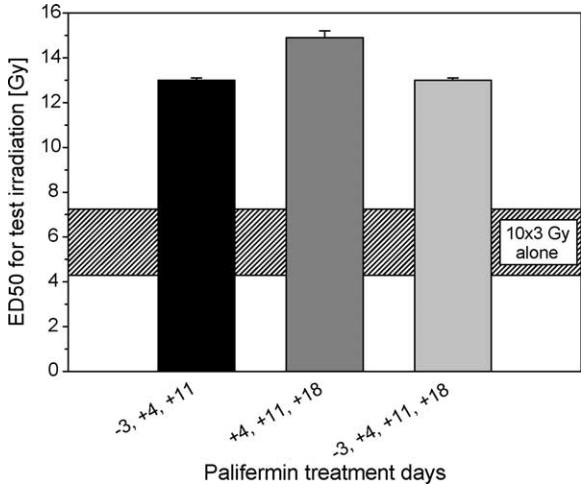

Fig. 3. Palifermin treatment over 3 or 4 consecutive weekends. KGF was applied subcutaneously on the days indicated at the abscissa. Error bars represent the standard deviation σ of the ED50-values, as computed by probit analyses. The hatched area reflects the ED50±σ for test irradiation after 10×3.0 Gy in 2 weeks without administration of palifermin.

palifermin administration, and no systematical variations were found with the palifermin administration protocols (data not shown).

Discussion

Radiotherapy of advanced head-and-neck tumours is usually associated with confluent oral mucositis as a severe early side effect, which frequently necessitates a treatment break [22,38]. Such prolongation of the prescribed overall treatment time for squamous carcinoma, however, can significantly decrease tumour control rates [3,38,40]. Moreover, severe early mucosal changes cause substantial impairment of the epithelial barrier function, and, as a result of additional trauma to underlying tissues, can aggravate late effects in form of a consequential component [1,5,14,25]. Therefore, patients may have a dual benefit from prophylaxis or treatment of the acute mucosal effects of radiotherapy.

A number of approaches for the management of early oral mucosal radiation effects have been tested both preclinically and clinically [11,23,26,29,39]. Most of them were symptomatic, aiming at reduction of secondary infections by topical antiseptic or antibiotic treatment. Some strategies, however, also encompassed administration of various growth factors [20,30,34,35,41]. However, so far no effective treatment has been identified in clinical studies and none of the approaches has found entrance into clinical routine of supportive care for head-and-neck cancer patients undergoing radiotherapy.

Recombinant human keratinocyte growth factor, palifermin, stimulates proliferation, differentiation and migration in epithelial cells in a variety of tissues, including the mucosal lining of the upper gastrointestinal tract. Hence, this growth factor appears a good candidate for amelioration of early radiation-induced mucositis. This has been shown in early studies for oral cavity, oesophagus and intestine [7,15,16,18,19] in mice.

In mouse tongue mucosa, ulcer frequency after single dose irradiation was significantly decreased by palifermin [15], with an increase in isoeffective doses by factors between 1.7 and 2.3. In combination with fractionated irradiation, a highly significant reduction in the incidence of mucosal ulceration was observed with various palifermin administration protocols [17]. In some protocols, even overcompensation of the entire fractionated dose was observed. A similar effect was found when palifermin was added to combined radiochemotherapy with cisplatinum and 5-fluorouracil (Dörr et al., submitted). Recently, a dose–effect relationship for palifermin doses was described between doses of 1 and 15 mg/kg (Dörr et al., submitted). However, even the lowest dose of 1 mg/kg produced a significant amelioration of the radiation-induced oral mucosal effect. These studies with fractionated irradiation suggested that palifermin given either during the weekend before the onset of irradiation or over the treatment-free weekend gaps is most effective. Therefore, the present study was initiated to investigate, with standard palifermin doses of 5 mg/kg, if variation of the timing of palifermin administration within the weekend gap would affect the effect, and further, if repeated applications over consecutive weekend gaps would potentiate the effect. The mouse tongue model was employed in all these experiments, using mucosal ulceration as the clinically most relevant endpoint.

All palifermin administration protocols tested in the present investigation resulted in a significant increase in isoeffective radiation doses for test irradiation after 2 weeks of fractionation, indicating that the residual tissue tolerance at this time was clearly increased. The numbers of radiation dose fractions compensated by palifermin, calculated on a linear–quadratic basis with an α/β-ratio of 11 Gy [10], according to the method described in Dörr et al. (submitted), varied between 4.6 and 6.9. This is in good agreement with previous findings [7,16]. This analysis proves that palifermin does not interfere with repopulation processes, which in mouse oral mucosa starts at the end of the first treatment week [6,8]. Impairment of effective repopulation, i.e. the regeneration response induced by fractionated radiotherapy, which is one of the major factors of radiation tolerance in oral mucosa, by growth factor treatment, would be clearly detrimental.

The present study revealed that the timing of the administration of palifermin within the weekend does not impact on the efficacy of the growth factor. For practical, clinical aspects, this illustrates that one injection of palifermin may readily be administered on the Friday of a treatment week, after the last radiation fraction, with an effect identical to three injections over the entire weekend.

In the present study, application of palifermin over 3–4 consecutive weekend gaps did not increase the effect over that of only two injections on two consecutive weekend gaps. This is in accordance with recent studies (Dörr et al., submitted), where optimally effective doses of palifermin at only one weekend gap (day -3, $+4$ or $+11$) were as effective as a combination over 2 or 3 weekends.

Recently, hypotheses of the mechanisms underlying the effect of palifermin have been discussed [7,16]. It was speculated that the pre-irradiation effect, but also partially the efficacy of palifermin given during radiotherapy, may be based on stimulation of stem cell proliferation, yielding more stem cells and also more differentiating cells. Moreover, stimulation of residual proliferation of doomed cells was suggested, which might enable damaged cells to undergo a higher number of abortive divisions, thus yielding higher overall cell numbers when radiotherapy is continued. The higher number of cells might then be able to counteract cell loss for a longer time, and might hence provide longer intervals for stem cell repopulation to become effective without the development of clinically manifest ulcer.

Safety of palifermin administration has recently been demonstrated; minor side effects in skin and plasma enzyme levels have been observed [4,7,33,36]. Clinical results of amelioration of oral mucositis by palifermin have recently been reviewed [7]. A phase I/II trial [2] in 60 patients resulted in shortening of the duration of confluent oral mucositis from 11 to 6.5 days. A placebo-controlled, randomized phase II study in 129 patients with hematological malignancies treated by total body irradiation and chemotherapy for bone marrow ablation [36] revealed a duration of mucositis grade 3–4 in the placebo group of 7.7 days, compared to 4–5 days in the palifermin groups

($P<0.05$). Recently, Spielberger et al. [37] reported results of a double-blind placebo-controlled randomized phase III trial of bone marrow ablation by a similar treatment protocol in 212 patients. Palifermin yielded less opioid analgesics and less total parenteral nutrition. A significant reduction in the incidence of oral mucositis WHO grade 3–4 (63 vs. 98%, $P<0.001$) and of grade 4 alone (20 vs. 62%, $P<0.001$) was found. Furthermore, palifermin significantly decreased mucositis-associated clinical sequelae.

None of the preclinical investigations [21,27] revealed any tumour-sparing activity of palifermin administration. However, conclusive pre-clinical data, using tumour cure as the relevant endpoint in clinically relevant fractionation protocols, are missing. The clinical data available so far do not indicate any tumour effect of palifermin, but the power of the studies does not allow for definitive statements on the tumour activity of palifermin.

In conclusion, palifermin represents an effective strategy to reduce oral mucosal side effects of radiation treatment in murine models. The potential of palifermin for amelioration of oral mucositis associated with radiotherapy of head-and-neck tumours has to be proven in well-designed, randomised, double blind clinical studies, with sufficient patient numbers. The palifermin administration protocols for these studies can be optimised based on the available preclinical data. On this basis, administration at the beginning of the treatment-free weekends, starting before the onset of radiotherapy, is recommended. However, further investigations into the mechanisms underlying the efficacy of palifermin to ameliorate oral mucositis are required for further optimisation of the administration protocols.

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