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European Journal of Cancer 38 (2002) 594–601

European Journal of Cancer

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Therapeutic outcome and side-effects after radiotherapy, chemotherapy and/or hyperthermia treatment of head and neck tumour xenografts

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Received 28 June 2001; received in revised form 26 October 2001; accepted 27 November 2001

Abstract

The aim of the study was to optimise the still unsatisfactory therapeutic results in head and neck cancer by studying the results and the side-effects of radiotherapy, chemotherapy and/or local hyperthermia treatment of human tumour xenografts. Mice carrying human-derived head and neck squamous cell carcinoma xenografts with a mean volume of 100 mm^3 received 5×2 Gy, cisplatin or ifosfamide and/or local hyperthermia at 41/41.8 °C. Haematocrit and tumour volumes were determined two or three times per week, respectively, until day 25 or day 60. At day 60, the highest number of complete remissions (CRs) (80%) was observed in the triple modality therapy group with radiation, local hyperthermia at 41.8 °C and cisplatin at a dosage of 2 mg/kg body weight (b.w.). Therapeutic side-effects were moderate weight loss and a mild anaemia. Thus, with regard to the long-term tumour-free survival, the most effective treatment was the combination of radiotherapy, cisplatin and local hyperthermia at 41.8 °C.
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Keywords: Head and neck cancer; Tumour xenograft; Radiotherapy; Chemotherapy; Local hyperthermia

1. Introduction

Despite multimodal treatment strategies for squamous cell carcinoma of the head and neck (SCCHN), the therapeutic results are still unsatisfactory. Since in clinical studies the general conditions cannot be effectively controlled and the treatment regimen can not be freely varied, we conducted animal studies on xenografted human head and neck tumours growing in nude mice. Conventional multimodal treatment strategies in the treatment of SCCHN are the sequential application of surgery followed by radiotherapy and/or chemotherapy (some weeks after surgery). However, radical surgery is often mutilating and radiotherapy and chemotherapy may not be applicable due to exclusion criteria. To improve treatment efficacy during the past decade, investigators have studied new combinations of widely employed single treatment modalities.

Hyperthermia, in combination with radiation and chemotherapy, is a new approach for these malignancies. The basis of this concept (triple modality therapy) is the simultaneous application of chemotherapy and hyperthermia directly after the radiation. The advantage of treatment combinations containing hyperthermia results from the supra-additive enhancement of the individual therapeutic effects, without an increase in the side-effects [1–3].

To study the treatment efficacy of different treatment modalities and their relationship to tumour-specific parameters (tumour volume, anaemia and metastatic spread), we conducted the following study on human-derived head and neck squamous cell tumour xenografts growing in nude mice.

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PII: S0959-8049(01)00411-7

2. Materials and methods

2.1. Tumours

Small pieces (volume 1 mm^3) of a human-derived head and neck squamous cell carcinoma (originally obtained from a patient with SCCHN, Department of Maxillo-facial-surgery, Medical University of Lübeck) were transplanted subcutaneously (s.c.) into the dorsum of the right hind paw of female athymic nude mice. Histologically, the tumour was characterised as a moderately differentiated squamous cell carcinoma. The tumour line was maintained as a serially transplantable tumour in nude mice. Female nude mice (nu-nu, 6–8 weeks old, weight 20–30 g) were kept in laminar-air-flow installations. Animals were allowed free access to food and water. All experiments were approved by the regional animal ethics committee and were conducted according to German federal law. Treatment was started when the tumours had reached a volume of approximately 100 mm^3 (after approximately 3 weeks).

2.2. Treatment protocol

The mice were divided into eight therapy groups and one control group (R0H0C0) of 15 mice each. Each therapy group received a different treatment regimen (Table 1). Table 2 shows the time schedule of the different treatments and measurements.

2.3. Radiotherapy

Tumour-bearing legs of mice were irradiated with 10 MeV photons (2 Gy per dose on 5 consecutive days) generated by a linear accelerator (Mevatron®, Siemens Corp.) at a dose rate of 3 Gy/min. The distance between the focus and isocentre was 100 cm. Field sizes of 10×10 cm^2 were used. Leaving the tumour site uncovered, the animals were shielded from the radiation by a 10-cm thick metal block. Perspex (Ø 2.5 cm) as a bolus material was placed in the radiation beam just above the tumours to shift the maximum dose to the surface of the tumours and to compensate for the build-up region. The field homogeneity was repeatedly checked using TLD dosimeters. During radiotherapy, the mice were narcotised with ether. The total radiation doses were 5×2 Gy and 10×2 Gy (20 Gy in group R2 only).

2.4. Hyperthermia

The mice treated with local hyperthermia (on the first day of the treatment directly after the first radiotherapeutic session) were placed for the treatment time of local hyperthermia (60 min) in specially constructed cages, where the water level could be adjusted such that only the paws (and thus the tumour) were submerged [4–6]. In this cage, the mice could move around freely. Under these conditions, it was not necessary to narcotise the mice. Only at the start of hyperthermia did the mice show signs of thermal discomfort. However, after a short time of adaptation, no signs of discomfort or pain were obvious. The animals were calm and even accepted food.

Target temperatures of 41 and 41.8 °C were chosen and applied for 60 min. 41 °C is often used as a mean temperature in clinically applied locoregional hyperthermia [7]. This also applies to 41.8 °C (60 min), which is used in whole body hyperthermia [8]. The water bath was mechanically agitated and thermostatically controlled to a temperature accuracy of ±0.1 °C (Thermomix 1420, B. Braun).

Previous measurements of intratumoral temperature (microthermocouple type K1/2; Philips, Munich, Germany) have shown a steady state of the intratumoral temperature within 4 ± 1 min to approximately 0.3 °C below the water bath temperature. The temperature of the water bath was therefore adjusted to 0.3 °C above the desired tumour temperature.

2.5. Chemotherapy

Cisplatin has been widely employed for the treatment of squamous cell carcinomas [9,10]. Ifosfamide is also active in squamous cell carcinoma and is part of multidrug protocols [11]. We chose cisplatin and ifosfamide

Table 1
Treatment components of the different groups

Groups (15 mice)	R1 (5×2 Gy)	R2 (10×2 Gy)	H1 (41 °C)	H2 (41.8 °C)	C1 (ifosfamide)	C2 (cisplatin)
R1	×					
R2		×				
R1H1	×		×			
R1H2	×			×		
R1H1C1	×		×		×	
R1H2C1	×			×	×	
R1H1C2	×		×			×
R1H2C2	×			×		×
R0H0C0 ($n=10$)						

R, radiotherapy; H, hyperthermia; C, chemotherapy.

because of their proven enhanced anticancer activity with increasing temperatures [12].

The mice of the respective groups received the cytotoxic drugs intravenously (i.v.), immediately after radiotherapy and before the start of local hyperthermia. The mice in the groups R1H1C1 and R1H2C1 received 32 mg/kg body weight (b.w.) ifosfamide in a concentration of 3.3 mg/ml. The activation time for ifosfamide in humans is approximately 30 min. Since the metabolic turnover of mice greatly exceeds that of humans, a drug activation time of 10 min was assumed in the mice. In order to ensure an effective therapeutic level of the activated drug at the beginning and during the 60-min period of hyperthermia, the first half of the total dose of 32 mg/kg b.w. ifosfamide was given 10 min before the start of the local hyperthermia treatment. The second half directly at the start of the local hyperthermia [2]. The groups R1H1C2 and R1H2C2 received 2 mg/kg b.w. cisplatin in a concentration of 0.16 mg/ml as a bolus at the start of the local hyperthermia. The cytotoxic drugs were injected i.v. by a catheter (Abbocath-T 26 G×19 mm) placed in the tail vein.

2.6. Volume measurements

The tumour diameter was measured three times a week with a conventional vernier caliper. The tumour volume was calculated from two diameters, measured at right angles to each other, using the formula for a half-ellipse ($a \cdot b^2$ ($\pi/6$)).

2.7. Haematocrit measurements

The haematocrit level was determined twice a week until day 25. After puncturing the tail vein, a small drop of blood was drawn into a microcapillary (9 μl) and put in a microcentrifuge (Compur M 1100).

2.8. Follow-up observation

To determine the long-term tumour-free survival, the animals were observed up to 60 days after the start of the experiment [13–15]. Complete remission (CR) was defined as the absence of tumour up to day 60. Partial remission (PR) was defined as a reduction of the tumour volume by at least 50% during the course of the treatment. Tumour progression was defined as the continuing growth of the tumour volume.

2.9. Histology

After 60 days, all livers and macroscopically suspect lungs were excised and prepared for light microscopy to evaluate the metastatic spread. Sections of 3 μm were cut on a microtome and stained with haematoxylin-eosin, as well as with trichrome.

Table 2
Time schedule of the different treatments and measurements

Days	1	2	3	4	5	6	7	8	9	10	11	12
RT	×	×	×	×	×			*	*	*	*	*
HT	×											
CT	×											
Hk	×				×			×				×
Tumour volume	×		×		×			×		×		×

RT, radiotherapy; HT, hyperthermia; CT, chemotherapy; Hk, haematocrit; * = 10×2 Gy in group R2 only.

2.10. Statistics

The Friedmann test was used as a statistical method to compare measurement results of the same group at different times. For the comparison of the groups with each other, the Kruskall–Wallis test was applied. For statistical analysis, the mean tumour volumes and the mean haematocrit values were calculated. Comparison between CR and PR was done using the Fisher's Exact probability test. Additionally, the 95% Confidence Interval (CI) for the number of CRs was calculated.

3. Results

3.1. Tumour volume (Fig. 1)

On day 1, the tumour volume of all treatment groups lay between 87.3 and 96.5 mm^3. In all treatment groups, the volume increased slightly until day 6, probably due to a therapy-induced tissue oedema. Subsequently, the tumour volume decreased steeply and reached the lowest level at day 25. On day 25, values lay between 1.75 and 31 mm^3. In all treatment groups, the mean tumour volume decreased at least by 50% (see Fig. 1, $P \leqslant 0.001$), i.e. at least a PR was achieved. The decrease in the tumour volume followed the same time course in all of the therapy groups. The differences of the mean tumour volumes between the different treatment groups were small and not significant. Significant differences were seen between the control group and all treatment groups ($P \leqslant 0.001$).

After reaching their lowest levels, the tumour volumes in all of the treatment groups increased again. This increase was caused by those tumours that responded only partially. Such tumours were found in each treatment group, but in differing numbers. Only in the control group (R0H0C0), did the tumour volumes increase continuously (progressive disease), ($P \leqslant 0.001$). Because the tumour volume at day 30 in the majority of mice in the control group had reached or exceeded 350 mm^3, all the mice in this group were killed for ethical reasons.

3.2. Anaemia

Fig. 2 shows the mean haematocrit values for the different treatment groups at different times during treatment. The mean haematocrit values of all groups on day 1 lay between 47 and 49.8 Vol.%. In all treatment groups, the haematocrit values decreased until day 8. The lowest haematocrit of 41.7 Vol.% was found in the triple modality group with radiotherapy (5×2 Gy), chemotherapy (ifosfamide) and hyperthermia (41.8 °C) (R1H2C1). After reaching the nadir, the haematocrit level increased again in all of the treatment groups. From day 15 to 25, the haematocrit values lay between 46 and 50 Vol.%. In all of the groups, the differences from the lowest haematocrit level to the highest hematocrit level were statistically significant within a range from $P \leqslant 0.05$ to $P \leqslant 0.001$. At day 25, the mice in all of the treatment groups had regained normal haematocrit levels.

In the group without therapy (R0H0C0), no change in the haematocrit level occurred. On day 1, the mean haematocrit was 47.5 Vol.% and on day 25, the mean haematocrit was 48.8 Vol.%. Thus, although the tumour volume increased steeply and reached values of 350 mm^3, the haematocrit values remained nearly unchanged.

3.3. Tumour remission and tumour-free survival at day 60 (Fig. 3)

The therapeutic efficacy was finally evaluated on day 60. The lowest number of CRs was found in the radiotherapy group (5×2 Gy), where a CR was achieved in

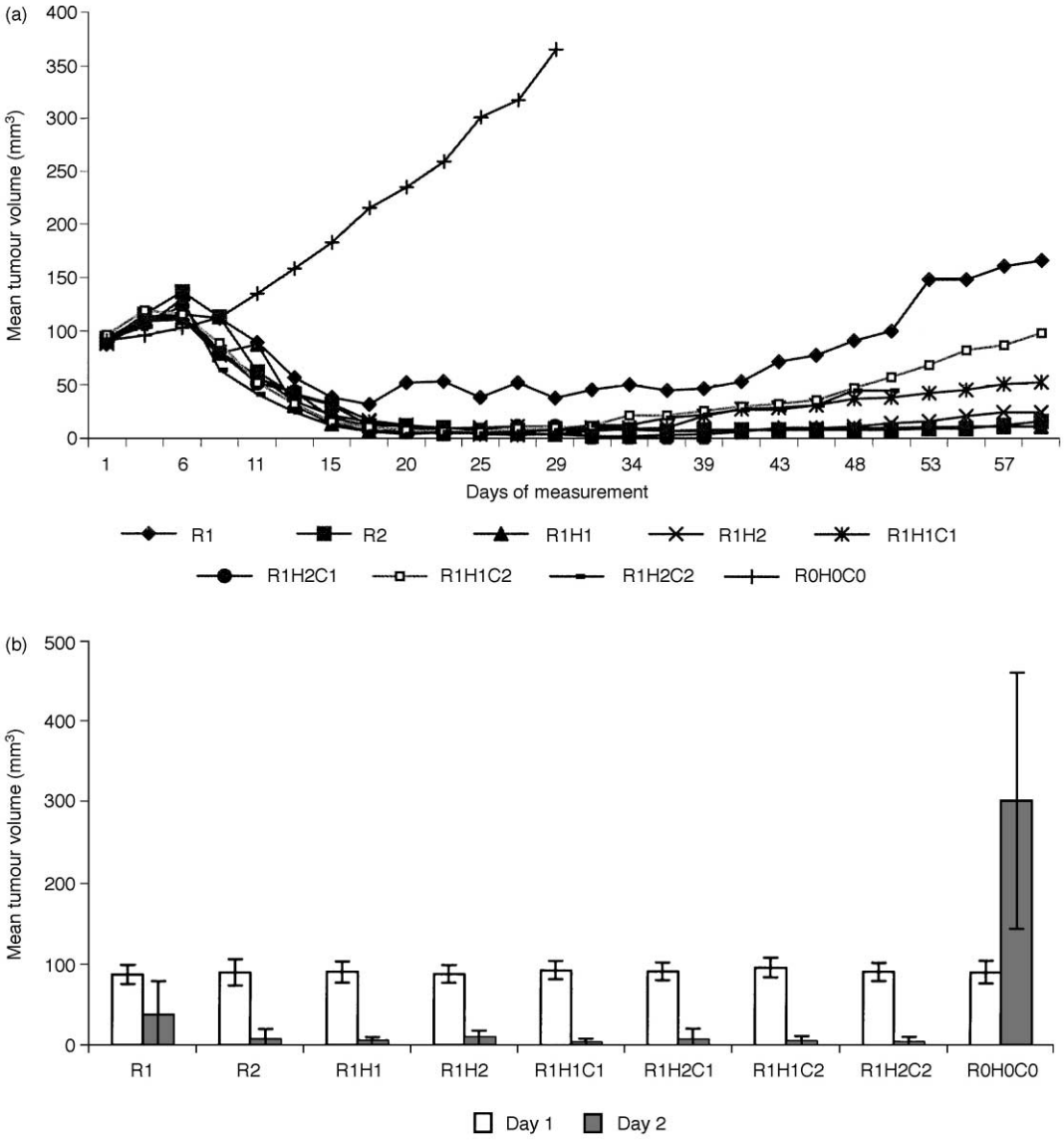

Fig. 1. (a) Mean tumour volumes of all groups (for clarification, no standard deviations are shown (range of SD: R1: 11.9–106.5; R2: 12.1–53.5; R1H1: 2–27.5; R1H2: 8.6–33.9; R1H1C1: 4.3–60.3; R1H2C1: 11.9–37.5; R1H1C2: 6.3–109.9; R1H2C2: 8.2–76.6; R0H0C0: 13.8–215.7)). (b) Mean tumour volumes of all groups on day 1 and day 25 (±SD).

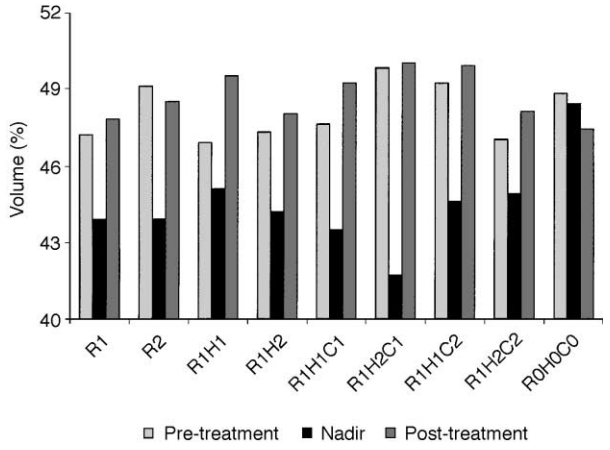

Fig. 2. Mean haematocrit levels at different times during therapy for all groups. For clarification, no standard deviations are shown (the range of standard deviation in all groups lay between 0.8 and 5.3). Pre-treatment = day 1, nadir = day 8, post-treatment = day 25.

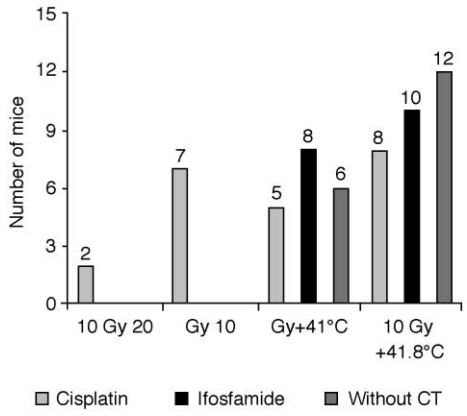

Fig. 3. Tumour-free survival at day 60. CT, chemotherapy.

only two mice (95% CI: 2–40). The increase of the radiation dose (10×2 Gy) in the group R2 led to a CR rate of seven out of 15 mice (95% CI: 21–73). A radiation dose of 20 Gy (10×2 Gy) alone was just as effective as a dose of 10 Gy (5×2 Gy) combined with hyperthermia 41.8 °C. The CR rate (5/15 mice) of the radiotherapy group with 5×2 Gy and hyperthermia at 41 °C (R1H1) lay in between the rate for the two radiotherapy alone regimens (95% CI: 12–61). The additional application of chemotherapy further improved the rate of CRs. In comparison with the R1H1 group (five mice with a CR), eight mice in the R1H1C1 group (5×2 Gy + 41 °C + ifosfamide) achieved a CR (95% CI: 27–78). In the R1H1C2 group (5×2 Gy + 41 °C + cisplatin) CR was found in six mice (95% CI: 16–67). This shows a higher efficacy of ifosfamide at a temperature of 41 °C compared with cisplatin at the same temperature. In addition, 41.8 °C, the additional chemotherapy improved the therapeutic outcome. The highest number of CRs (12 mice, 80%) was observed in the triple modality group with radiation (5×2 Gy), hyperthermia at 41.8 °C and cisplatin 2 mg/kg b.w. (R1H2C2) ($P \leqslant 0.001$, 95% CI: 52–95).

In all of the control mice (R0H0C0), the disease progressed throughout the time of observation (95% CI: 0–30).

3.4. Side-effects

The single and combined therapies were generally well tolerated. Side-effects were mild in all of the groups. Nearly all of the mice lost approximately 10% of their body weight during the course of treatment, However, they regained their initial body weight a few days after the end of therapy. Anaemia was present in all mice. The haematocrit level dropped approximately 15% below physiological values during the course of treatment.

Two weeks after the end of therapy, the haematocrit values returned to normal levels (see Fig. 2). There was no correlation between the decrease of haematocrit and the long-term tumour-free survival.

Acute cutaneous side-effects like thermal blisters or ulcerations were absent. None of the mice showed signs of discomfort or pain during and after the treatment sessions. Only one mouse from the group receiving radiotherapy, hyperthermia 41 °C and chemotherapy with ifosfamide (R1H1C1) developed severe side-effects. Two days after the start of therapy, the mouse showed signs of neurotoxicity. Generalised seizures were the main symptom. Anticonvulsive therapy with a short ketamine anaesthesia (0.5 mg/kg b.w. i.v. as a single dose) stopped the seizures.

3.5. Histology

At the end of the experiment, no metastatic spread was observed—all livers and lungs were tumour-free, even in the untreated animals.

4. Discussion

This study was performed to evaluate the feasibility, efficacy and acute toxicity of single and combined tumour treatment protocols. Our main purpose was to compare different clinically used tumour treatment modalities and, especially, various triple modality regimens with regard to therapeutic efficacy and outcome.

Numerous clinical studies have confirmed that a combination of radiation, hyperthermia and chemotherapy (triple modality therapy) leads to a higher therapeutic efficacy [1,2,9]. *Triple modality therapy* means the (practically) simultaneous application of hyperthermia and chemotherapy directly after the radiation. This concept is different from the *conventional multimodal therapy* where the different treatment modalities are often applied sequentially, sometimes with a time interval of weeks.

Triple modality therapy has been examined in a number of studies. In an extensive review, Herman and colleagues (1988) presented the available data up until 1988 [16]. A more recent review regarding triple modality therapy was published by Feyerabend and colleagues in 1997 [17].

Overgaard [18] was the first to report on the use of misonidazole (1 mg/g), hyperthermia and radiation as treatment for local animal tumours (C3H mammary carcinoma in mice). He observed a 3-fold increase in therapeutic gain when the three treatment modalities were applied simultaneously. Kai and colleagues [5] combined bleomycin (34 mg/kg) with hyperthermia and radiation in the Ehrlich ascites tumour transplanted into mice and found a significant decrease in the rate of tumour growth when triple modality therapy was used versus any of the double modality treatments.

Herman and Teicher (1988) evaluated the use of cisplatin (5 mg/kg) with heat and radiation in FSaIIC fibrosarcomas transplanted into mice. They found that the use of this trimodal therapy produced strikingly longer tumour growth delays than any single or double modality treatment [19].

In 1989, Hou and colleagues [20] were the first to introduce triple modality tumour therapy for patients with oesophageal cancers. Thereafter, Amichetti and colleagues, Feyerabend and colleagues, Steindorfer and colleagues, Dahl and colleagues and others have performed various clinical studies confirming that triple modality therapy substantially improves the clinical results [1–3,9]. A highly relevant study for comparison with this study was performed by Herman and colleagues [21]. They carried out a phase I-II study with the same treatment modalities as were used in this study (cisplatin 20/30/40 mg/m^2, hyperthermia and radiation). Tumour types in their study were SCCHN and chest wall/breast adenocarcinoma. They determined the tolerable dose of cisplatin and estimated the therapeutic potential of this trimodal therapy. In accordance with our study, they found that the triple modality therapy with cisplatin, hyperthermia and radiation is highly effective and associated with low toxicity. In 50% of the patients, the combined therapy led to CRs, the other 50% responded with a PR. In our study, we found nearly the same ratio for CR/PR.

In our preclinical study, experiments on triple modality therapies were carried out for the first time with head and neck cancer xenografts in this special experimental setting. In accordance with the data in the literature, we found that, in terms of long-term tumour-free survival, the triple modality therapy is more effective than single or double modality treatments.

4.1. Tumour remissions and tumour-free survival

We observed significant differences in the long-term tumour-free survival rates between the various treatment regimens. Especially the treatment combination of radiotherapy, hyperthermia at 41.8 °C and cisplatin was highly effective in the therapy of head and neck tumours. The comparison of the triple modality regimen with cisplatin and ifosfamide shows that the combination of radiotherapy, hyperthermia at 41.8 °C and cisplatin is more effective than the same combination with ifosfamide. Hyperthermia enhances the activity of cisplatin as well as of ifosfamide. However, the temperature optimum seems to differ [1,12]. At a tissue temperature of 41 °C, ifosfamide is more effective than cisplatin, whereas at 41.8 °C the effect of cisplatin exceeds that of ifosfamide. From our results, it therefore appears that the temperature optimum of cisplatin is thus higher than that of ifosfamide.

In our experiments, all tumours had a volume of approximately 100 mm^3 at the start of therapy. Under different treatment protocols, the tumour volumes initially decreased rapidly, but there were no significant differences between the treatment groups. Radiotherapy alone induced at least a PR. Although the time course of volume change was similar, the rate of CRs was different between the groups.

4.2. Treatment side-effects

The side-effects of the single and combined therapies in our study were mild anaemia and moderate weight loss. In various experimental and clinical studies, similar observations have been made. Baker and colleagues [22] who evaluated the acute skin reactions in mice elicited by a combination therapy with cisplatin (6 mg/kg), heat and radiation did not find an increase in normal skin reactions with their trimodal therapy compared with heat and radiation alone. Redpath and colleagues [23] also found no increase in acute skin reactions in mice when doxorubicin (10 mg/kg) was added to heat and radiation.

Amichetti and colleagues [1] evaluated the toxicity in 18 patients with SCCHN treated with radiotherapy, chemotherapy (cisplatin 20 mg/m^2) and hyperthermia and found an acceptable toxicity profile (dry skin desquamation, moderate haematological toxicity, mild nausea).

Planting and colleagues [10] noted mild anaemia, nausea, mild to moderate nephrotoxicity, neurotoxicity, ototoxicity and weight loss in a phase II study of weekly high-dose cisplatin (80 mg/m^2) in patients with advanced SCCHN.

Kurowski and Wagner [24] studied the influence of ifosfamide (1.5 g/m^2) chemotherapy in 11 patients with lung cancer. They found a Central Nervous System (CNS)-toxic compound of ifosfamide (chloroacetaldehyde), which is released in the alternative metabolic pathway from ifosfamide. Recent reports indicate that a characteristic, severe, but reversible, ifosfamide-

associated encephalopathy may occur, particularly in children [25,26]. In our study, one mouse showed signs of CNS toxicity, although the ifosfamide dose was relatively low.

Many clinical studies have confirmed that anaemia has a significant influence on survival in patients treated with different protocols. For example, Dubray and colleagues [27] showed that local tumour control is significantly lower in patients (SCCHN in 217 patients) with pretherapeutic anaemia (haemoglobin level below 135 g/l in men and below 120 g/l in women) compared with non-anaemic patients. In accordance with that, Fein and colleagues [28] reported a 2-year disease-free survival of 88% (in 109 patients with SCCHN), when the pretreatment haemoglobin level was >130 g/l, in contrast to a survival rate of 46% when the pretreatment haemoglobin level was <130 g/l. Tarnawski and colleagues [29] observed that the haemoglobin level (in 971 patients with SCCHN) at the start of radiotherapy did not correlate with treatment outcome, but any decrease of haemoglobin during therapy was a strong prognostic factor for treatment failure.

In our experiments, no mouse was anaemic at the start of therapy. However, during therapy, all of the mice in the treatment groups developed anaemia. Interestingly, in spite of rapid tumour progression, none of the mice in the control group developed anaemia. We found that therapy-associated anaemia alone had no influence on outcome. This is not in accordance with the above-mentioned studies.

One explanation for the time course of haematocrit in our study may be the lower dose of radiotherapy and chemotherapy compared with tumour therapy given to humans. The lower radio- and chemotherapy doses applied in our study reduced the haematocrit, but the suppression of the haematopoetic activity was mild and in our mice the bone marrow was not apparently damaged irreversibly.

In all of the clinical studies, higher rates of side-effects than those observed in our study were reported. This again could be due to the higher dose of radiotherapy and chemotherapy used in the clinical studies. The total doses of radiotherapy used clinically are significantly higher than those in our study; however, the applicable dose was limited because radiation rates of 20 Gy led to a complete tumour regression in approximately 50% of the treated animals within 60 days. Since we were interested in studying the long-term efficacy of the combined therapy, we had to apply lower total radiation doses.

Furthermore, in our study, the follow-up time of 60 days was short for the evaluation of late complications.

In conclusion, the results of our study show that triple modality therapy is associated with relatively low toxicity and is a highly effective option for the treatment of head and neck cancer xenografts transplanted into nude mice. The combination of radiotherapy, cisplatin and hyperthermia (at 41.8 °C) in our experimental set-up leads to CR rates of up to 80%. This seems to be the best combined therapy for SCCHN as single treatment options and double combined therapies led to CR rates of only 13 and 53%, respectively.

We observed no difference in the frequency and severity of side-effects associated with the single, double and triple combinations of tumour therapy.

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