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ORIGINAL PAPERS

Few X-ray and PUVA treatments accelerate photocarcinogenesis in hairless mice

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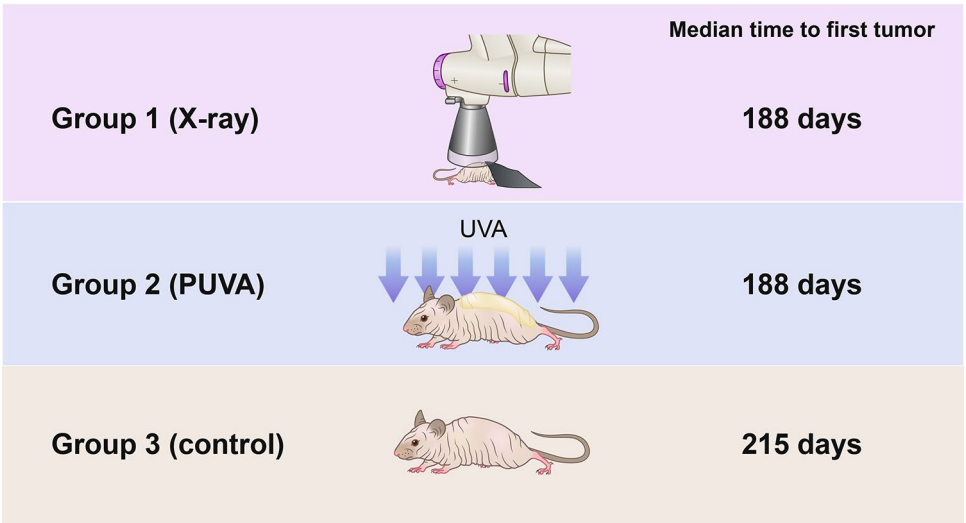
Abstract

PUVA is a treatment that combines oral methoxypsoralen (8-MOP) with ultraviolet radiation A (UVA). It is used for severe psoriasis and the early stages of T-cell lymphoma. X-rays are an effective treatment for skin cancers. Both treatments are in higher doses used to treat skin malignancies and simultaneously increase the risk of keratinocyte cancer. The main objective of this study was to test whether a few PUVA or X-ray treatments could delay the development of ultraviolet radiation (UVR)-induced skin tumors in a well-established hairless mouse model. Three groups of immunocompetent mice (total, $N = 75$) were included in the study. All groups were UVR-exposed during the study period. In addition, one group was treated with PUVA and another group was treated with X-rays at days 45, 52, 90 and 97. A control group was treated with UVR only. We recorded when the first, second and third skin tumors were induced in each mouse. Skin tumors developed significantly earlier in both the PUVA and X-ray groups (median, 188 days) than in the control mice (median, 215 days; $p < 0.001$). Therefore, a few X-ray and PUVA treatments both significantly accelerated the development of skin tumors in hairless mice, compared to UVR controls. Neither treatment showed a delay of UVR-induced skin tumors and caution should be exercised before applying these treatments to sun-damaged skin.

Graphic abstract



Keywords 8-MOP · PUVA · Radiation therapy · X-ray · Ultraviolet radiation · Skin tumors · Prophylactic treatment · Hairless mice

Extended author information available on the last page of the article

1 Introduction

Radiation therapy using X-rays has been used as a treatment for skin cancer for a century. It is well known that ionizing X-ray radiation can cause keratinocyte cancers but can also be used to kill cancer cells [1–3]. The use of X-rays in dermatology has decreased over the past few decades, probably due to awareness of the hazards of ionizing radiation and the introduction of other treatments [4, 5]. X-rays are still used to treat skin tumors such as squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs), as well as cutaneous lymphomas and other primary skin cancers [6, 7]. The mechanism of action involves X-rays altering DNA and causing chromosomal aberrations that inactivate cell division [8, 9]. Rapidly dividing cells are particularly susceptible to destruction by radiation [9]. Moreover, X-rays damage Langerhans cells in the epidermis of both humans and mice, producing anti-inflammatory responses [8, 10, 11]. However, X-rays can elicit immunosuppressive effects that may increase the sensitivity of skin to ultraviolet radiation (UVR), promoting carcinogenesis [12].

8-Methoxypsoralen (8-MOP) is a naturally occurring furanocoumarin that may be combined with ultraviolet radiation A (UVA) to treat skin diseases [13]. This treatment combination is called PUVA or photochemotherapy [13]. PUVA is most frequently used to treat severe psoriasis and mycosis fungoides. However, several studies have reported that PUVA has a carcinogenic effect on patients with psoriasis [14–18]. A large-scale epidemiological study by Lindelöf et al. confirmed that male patients who had received more than 200 treatments with PUVA exhibited an incidence of SCC that was more than 30-fold greater than in the general population [17]. On the other hand, PUVA may also induce prolonged disease-free intervals if used in the early stages of cutaneous T-cell lymphoma [19, 20].

In this study, we investigated whether a few treatments with X-rays or PUVA could delay the development of UV-induced skin tumors in hairless mice. We have previously shown that a few prophylactic treatments with photodynamic therapy can postpone tumor development both in mice [21, 22] and humans [23]. Therefore, we wanted to explore if there could be a prophylactic effect of a few treatments with X-ray and PUVA on photocarcinogenesis in mice.

2 Materials and methods

2.1 Animals

Three groups of 25 female C3.Cg/TifBomTac immunocompetent mice (total, $N = 75$) were included in the study and were 13–16 weeks old when the experiment was initiated. All mice were tattooed with consecutive numbers on the abdomen and each mouse group was housed in a separate box where there was free access to drinking water and standard laboratory food. They were kept on a 12-h light/dark cycle in a 23–24 °C warm facility. Treatment of the animals was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of The Danish Animal Experiments Inspectorate. The facility is screened at least once per year, in accordance with the Federation of Laboratory Animal Science Associations guidelines and no positive results for pathogens were found (Idexx BioAnalytics, Kornwestheim, Germany).

2.2 UV exposure

All mice were irradiated using three standard erythema doses (SEDs) three times per week from the start of the study [24]. The UVR source was composed of one UV6 tube (Waldmann™, Wheeling, IL, USA) positioned between five Bellarium-S SA-1-12 tubes (Wolff System™, Atlanta, GA, USA). The spectrum from the light source is described by Lerche et al. [25]. The animals were irradiated from above, with the UVR passing through the wire lid of each box. Adjustment of the distance from the light source to the animals was performed monthly to sustain the desired doses. The UV-dose was measured using a spectroradiometer (Solatell Sola-Hazard 4D Controls Ltd., Cornwall, UK). Typically, a Danish summer midday sun delivers 3 SED in 30 min [26].

2.3 Treatments

One treatment with X-rays or PUVA was given on each of days 45, 52, 90 and 97 (Fig. 1). The skin was not prepared in any way prior to the treatments. Group 1 mice received X-ray treatment with 20 kV (half-value depth of 2 mm skin) and a dose of 5 Gy for 1 min 19 s using an X-ray system normally used for patients in the dermatological clinic (model D3100; Gulmay Medical, Surrey, UK). The mice were anesthetized with 0.05 mL fentanyl citrate (0.158 mg/mL) + fluanisone (5 mg/mL) + midazolam (2.5 mg/mL) and protected with a lead shield over the head while receiving the treatment (Fig. 1). Group 2 mice received treatment with PUVA. Capsules containing 10 mg 8-MOP (G.L Pharma, Lannach, Austria) were opened and 100 µL was applied to an area of 15 cm^2 on the dorsal skin of the mice (from front legs to tail) corresponding to 2 mg 8-MOP per mouse. Next, 1 h after the topical application of 8-MOP, the mice were irradiated with UVA from above their cages. The UVA source consisted of 6 CLEO Performance 40W-R tubes (Philips, Eindhoven, The Netherlands) and the mice received 1 J/cm^2 given in 4 min

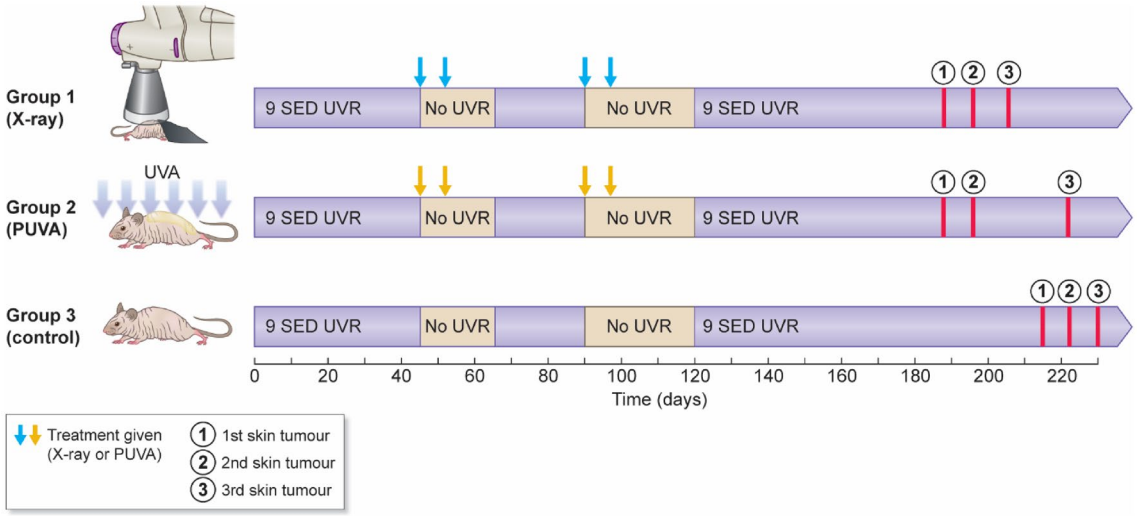


Fig. 1 Overview of treatment groups and study design. Mice received 3 × 3 SED of UVR per week. *SED* standard erythema dose, *UVR* ultraviolet radiation

and 36 s. The control group did not receive any treatment except for the thrice weekly UVR exposure.

2.4 Study design

Mice were examined for tumors weekly. The "time to the first tumor" was the number of days it took for the first 1 mm-diameter tumor to appear that later grew to a size of 4 mm [27]. As secondary endpoints, we also recorded the time to the second and third tumors based on the same criteria. The mice were killed according to protocol when they had developed three tumors with a diameter of 4 mm or after 365 days. Next, two of these mice were randomly selected from each group and one tumor from each mouse was mounted in Tissue-Tek OCT compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and frozen. Hematoxylin and eosin stained biopsies were sectioned vertically at 10-mm thickness and evaluated by a Mohs surgeon (Author, MG). Weight and skin pigmentation measurements were recorded monthly. Pigmentation was quantified in arbitrary units (au), based on the 20-point Kodak Gray Scale. The UVR control mice used here were also used in another study [22], but all trials were conducted simultaneously.

2.5 Statistics

We used both parametric and nonparametric statistics, including medians and percentiles for descriptive data. The times to onset of the first, second and third tumors were presented in Kaplan–Meier plots. Groups were compared using Mantel–Cox/log-rank tests. Differences were considered significant when p-values were < 0.05. All analyses were performed using IBM SPSS 25 for Windows (SPSS Inc., Chicago, IL, USA). Weight and skin pigmentation measurements were evaluated using one-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test for groupwise post-hoc comparisons.

3 Results

3.1 Clinical reactions to the treatments

The treatments used in this study are presented schematically in Fig. 1. All mice were exposed to UVR for a total of three times per week from day 0 until tumor development, with pauses from day 45 to day 66 and from day 90 to day 120. These UVR treatment pauses were due to local skin reactions in the PUVA group. On days 45, 52, 90 and 97, the relevant groups of mice were treated with X-rays or PUVA.

Mice treated with X-rays exhibited no signs of discomfort or skin reactions. Mice treated with PUVA developed erythema, crusting and scaling after treatments (Fig. 2). The dose was clearly supra inflammatory. The PUVA group had more intense and longer-lasting reactions after the second and fourth treatments, compared to the first and third treatments. The control group did not have any skin reactions before tumor development. No weight difference was observed among the groups ($p > 0.05$). Three mice in the X-ray group, three mice in the PUVA group and one mouse in the control group died before tumor development. This observation did not reveal any statistically significant differences ($p > 0.05$).

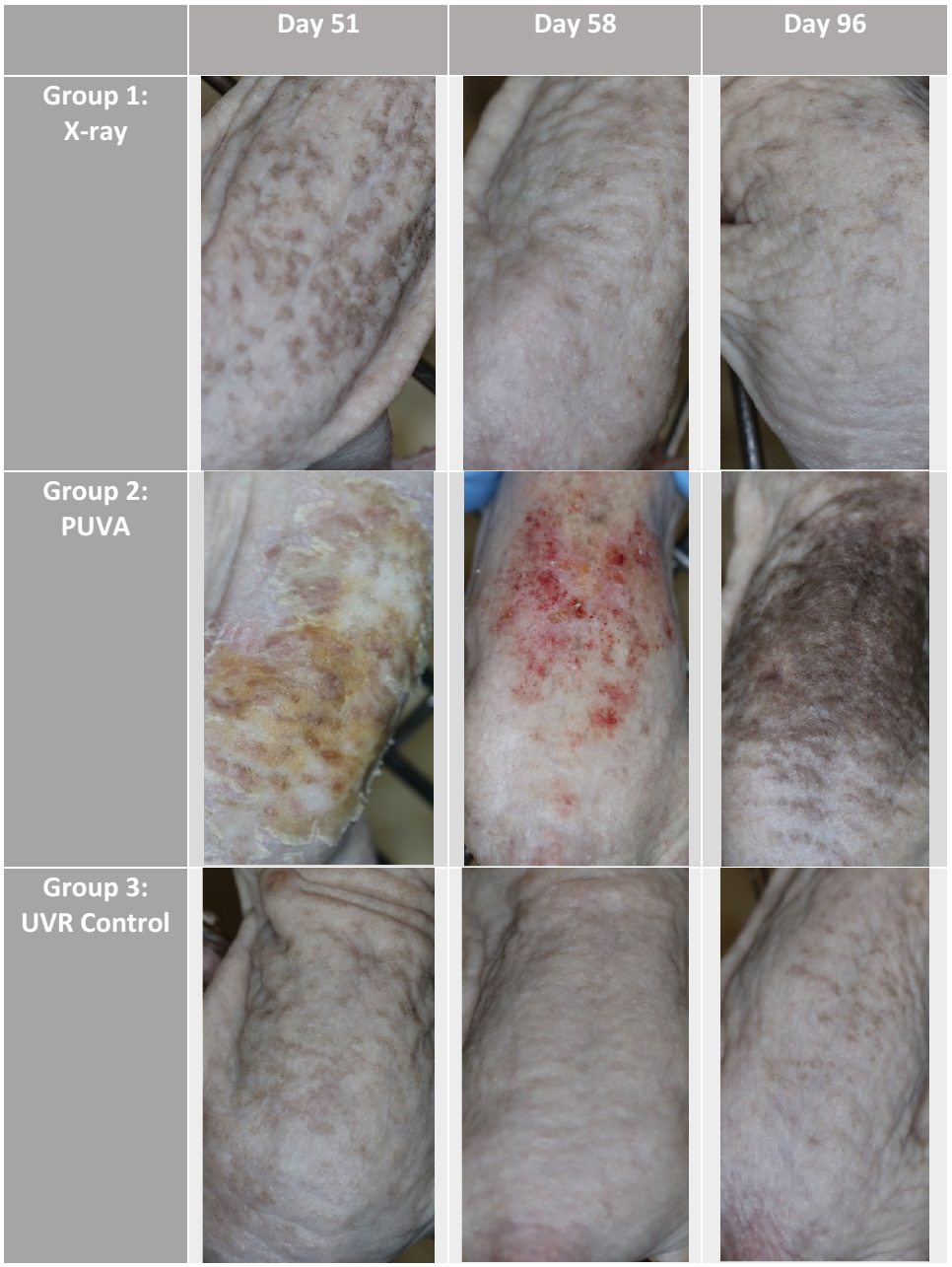


Fig. 2 Skin reactions at different times after treatments. Days 51, 58, and 96 are 6 days after Groups 1 and 2 were treated with the 1st, 2nd, and 3rd X-ray or PUVA treatment, respectively. In the PUVA group, erythema, crusting and scaling developed after the treatments; hyperpigmentation also developed thereafter

3.2 Treatment efficacy

All the mice developed at least one tumor. All tumors examined were SCCs (Fig. 3). Throughout the study, there was no noticeable evidence of general or internal disease that may have indicated the presence of internal cancers. Mice treated with X-rays and those treated with PUVA developed their first tumor significantly earlier than did control mice (188 vs. 215 days, $p = 0.000006$ and 188 vs. 215 days, $p = 0.000016$, respectively; Fig. 4 and Table 1). In both the X-ray and PUVA treated groups, the first tumors developed at the same times (188 vs. 188 days, $p = 0.973$).

Likewise, the second tumors developed significantly earlier in the mice treated with X-rays and those treated with PUVA, compared with the controls (196 vs. 230 days, $p = 3.6 \times 10^{-8}$ and 196 vs. 230 days, $p = 0.000022$, respectively; Fig. 4 and Table 1). In both the X-ray and PUVA treated groups, the second tumors developed at the same times (196 vs. 196 days, $p = 0.388$).

Last, the third tumors developed significantly earlier in the mice treated with X-rays and those treated with PUVA, compared with the controls (206 vs. 230 days, $p = 4.3 \times 10^{-8}$ and 222 vs. 230 days, $p = 0.001$, respectively; Fig. 4 and Table 1). The X-ray group developed

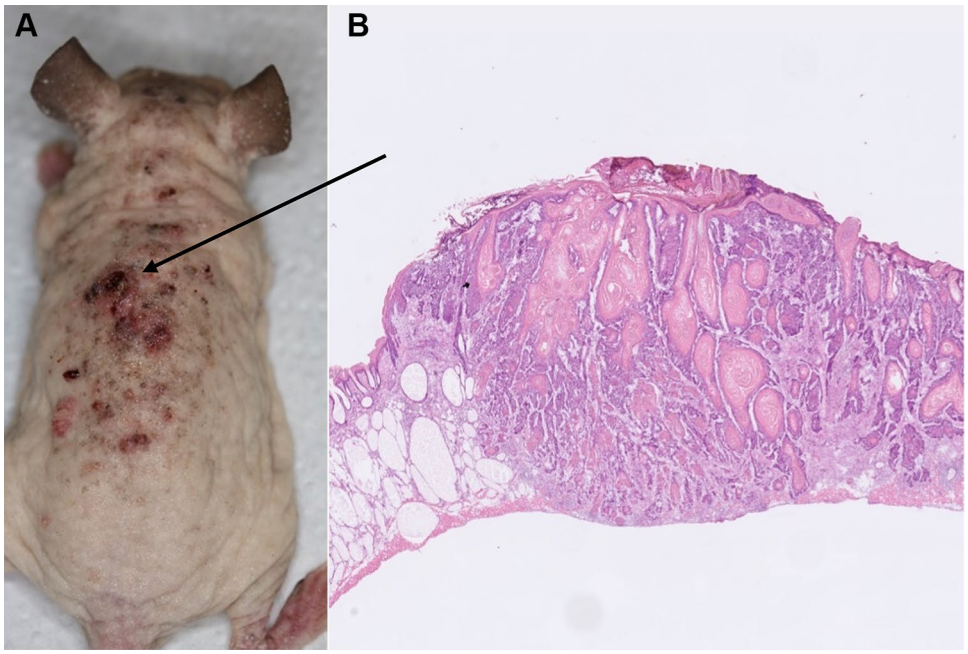


Fig. 3 (**A**) Representative mouse with squamous cell carcinoma (arrow). (**B**) Histology of squamous cell carcinoma from the mouse in 3A shows a moderately differentiated tumor (hematoxylin and eosin, × 2)

their third tumors slightly earlier than the PUVA group (206 vs. 222 days, $p = 0.005$).

3.3 Pigmentation

All mice were equally pigmented after UVR until the treatments started at day 45 (Fig. 5). At days 84 and 124 the PUVA treated mice were much more pigmented than the X-ray treated and control mice ($p < 0.00001$). The pigmentation of PUVA treated mice was not measured at day 60 due to erythema. The X-ray treated and UVR control mice showed similar pigmentation, with the exception of day 60 at which the X-ray treated mice were slightly more pigmented ($p = 0.002$).

4 Discussion

The present study investigated whether treating the skin of hairless mice with a few treatments of X-rays or PUVA could delay UVR-induced photocarcinogenesis. The treatments were scheduled at days 45, 52, 90 and 97 after UVR-exposure start. Resembling a possible prophylactic treatment on a slightly sun damaged skin after 1.5 months and 3 months of UVR-exposure. We have previous experience of delaying tumor development after prophylactic treatment with photodynamic therapy at days 45 and 90 [21, 22]. Therefore, this treatment schedule was chosen in this study.

In this murine model, we found that additional few treatments with X-rays or PUVA did not postpone the development of the first, second and third tumors, compared to mice that only received UVR. Both treatments proved ineffective in delaying UVR-induced skin tumors in hairless mice. X-ray and PUVA are both treatments used to treat diseases by killing cells and, therefore, theoretically there could have been a prophylactic effect of the few treatments with X-ray and PUVA. However, this study proved opposite and it is well known that cancer therapy (including radiation) is a double-edged sword. It is shown that the dead and dying cancer cells generated by chemotherapy and targeted cancer therapy paradoxically trigger inflammation that can promote aggressive tumor growth [28].

Mice treated with PUVA developed intense hyperpigmentation, measured on the Kodak gray scale; however, this did not delay the development of skin tumors compared to our control group. The PUVA treated mice had a small delay in time to development of the third tumor compared with the X-ray treated group. Hyperpigmentation could be the reason for that. The hyperpigmentation we observed in response to PUVA also occurs in patients [29]. In fact, the significant differences we observed between the groups indicate that these treatments have a co-carcinogenic effect, because tumors developed earlier in the X-ray and PUVA treated groups than in control mice that only received UVR. Co-carcinogenic reagents or treatments are not carcinogenic when applied in isolation but can increase the rate and size of tumors induced by other reagents or treatments (e.g., UVR)

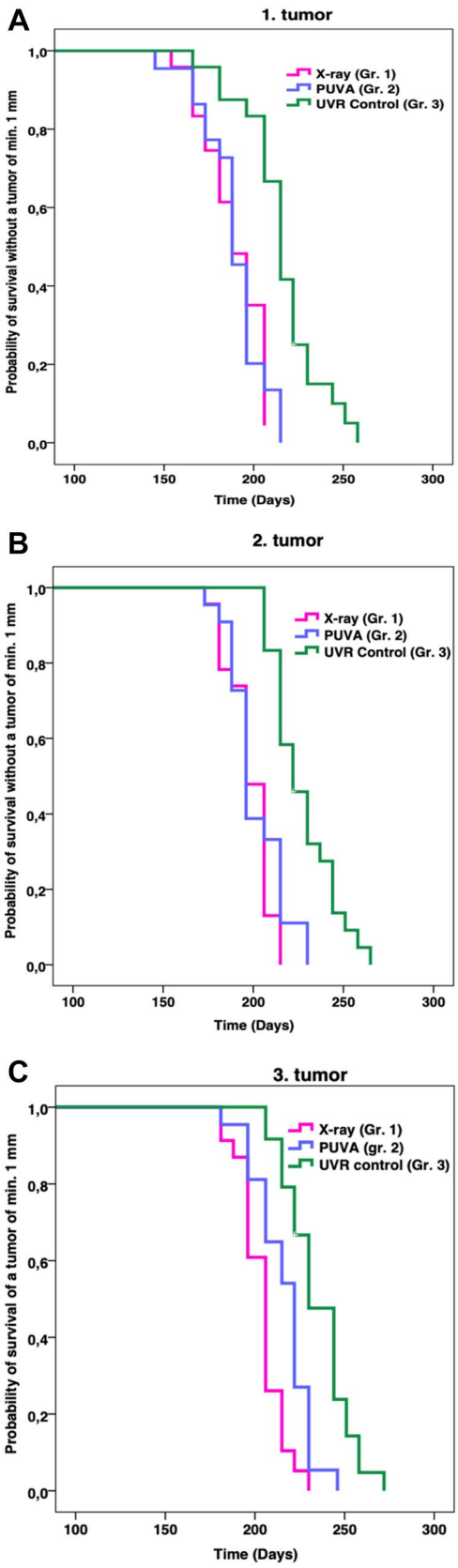


Fig. 4 Kaplan–Meier plots showing the probability of survival without a first, second and third tumor (minimum diameter = 1 mm) as a function of time

[30]. We would not expect the doses of X-rays or PUVA used in this study to be carcinogenic when applied in isolation; therefore, no mice were treated with X-rays or PUVA in the absence of UVR.

Previously, we demonstrated that a dose of 45 Gy using 50 kV of X-rays had a carcinogenic effect on the same strain of hairless mice used in this study [12]. Interestingly, in the present study we observed co-carcinogenic effects elicited by a total dose of 20 Gy using 20 kV, suggesting that this mouse strain is a sensitive model. This dose was chosen because a 5 Gy single dose does not induced visible inflammation in the mice and 20 kV reach the dermis (half-value depth of 2 mm full thickness human skin). It should be noted that Grenz rays (10–15 kV—approximately half-value depth of 0.25 mm full thickness human skin), also called ultrasoft X-rays or Bucky rays, are typically administered at doses of 1 or 2 Gy per session at weekly intervals for a total of 8–10 Gy [31]. A voltage of 10–15 kV will treat epidermis and a voltage of 20 kV will treat both epidermis and dermis. In this study, the voltage and dose were higher than the typical Grenz ray treatment.

In a study of 14,140 patients who had X-ray treatment for benign skin disorders, Lindelöf et al. found no association between the incidence of malignant skin tumors and accumulated X-ray doses of ≤ 100 Gy [1]. In contrast, a study by Karagas et al. that followed 5,232 participants over 6 years reported an increased risk of BCC after ionizing radiation therapy. The risk of developing BCCs apparently increased over time and was higher in those participants who were younger when treated [2]. Frentz et al. reported an increased risk of nonmelanoma skin cancers in patients who were treated with therapeutic doses of Grenz rays [32]. This is consistent with our results, which showed that X-rays accelerated the development of skin tumors.

Having up to approximately 200 PUVA treatments is generally considered "safe" [26]. However, because people are exposed to many different stimuli during their lifetimes, it is difficult to estimate the carcinogenic potential of PUVA in humans. In this study, we found that PUVA has a measurable co-carcinogenic effect after only a few treatments.

This study demonstrated that a few treatments of X-rays or PUVA did not delay the development of skin tumors in hairless mice. In contrast, even small doses of these treatments had statistically significant co-carcinogenic effects, compared to control a group. Therefore, neither X-rays nor PUVA can be used as a prophylactic treatment against UV-induced skin cancer.

Table 1 Median number of days to onset of the first, second and third tumors in the three groups. Interquartile ranges (25th and 75th percentiles) are shown

Group no.	No. of mice (*n*)	Treatment	Median days to 1st tumor	Median days to 2nd tumor	Median days to 3rd tumor
1	25	X-ray	188 (173–206)	196 (188–206)	206 (196–215)
		p value*	0.000006	3.6×10^{-8}	4.3×10^{-8}
2	25	PUVA	188 (181–196)	196 (188–215)	222 (206–230)
		p value*	0.000016	0.000022	0.001
3	25	Control	215 (206–222)	222 (215–244)	230 (222–244)

**p* value for the group in question is compared with that of the control group

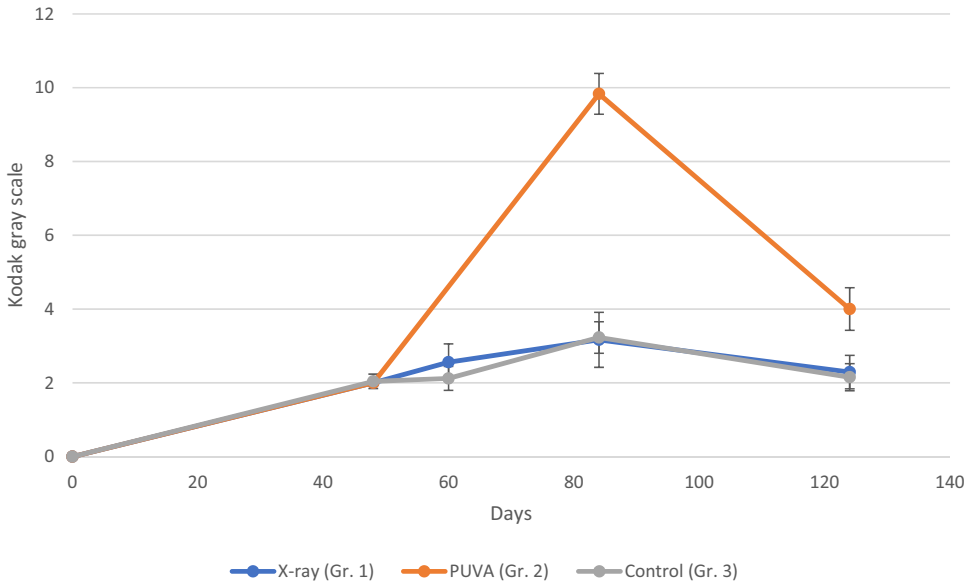


Fig. 5 Skin pigmentation measured on a Kodak Gray scale (arbitrary units, au) for all groups of mice. The steep rise in pigmentation indicates hyperpigmentation that developed after treatments. Hyperpigmentation was particularly marked at day 84 in mice treated with PUVA. The pigmentation present in mice treated with X-rays was not significantly different from that present in control mice ($p > 0.05$)

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Author contributions Conceptualization: HCW, PAP, and CML; methodology: HCW, PAP, and CML; validation: MG; formal analysis: PAP, CML, and MG; investigation: CML; resources: HCW and CML; data curation: RNA; writing—original draft preparation: RNA and CML; writing—review and editing: HCW, RNA, MG, PAP, and CML; visualization: RNA and CML; supervision: HCW and CML; project administration: CML; funding acquisition: CML. All authors have read and agreed to the published version of the manuscript.

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Data availability The data presented in this study are available on request from the corresponding author.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval All protocols were approved by the Danish Animal Experiments Inspectorate (permit number 2014-15-0201-00096) and our Institutional Animal Care and Use Committee.

Consent to participate Not applicable.

Consent for publication All authors have read and agreed to the published version of the manuscript.

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