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ORIGINAL ARTICLE

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Forphenicinol enhances the antitumor effects of cyclophosphamide in a model of squamous cell carcinoma

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Abstract We examined the interaction between forphenicinol (FPL) and cyclophosphamide (CPA) or ionizing radiation (IR) on the growth of murine squamous cell carcinoma tumors SCCVII. Primary tumors were established in C3H mice by injecting SCCVII tumor cells subcutaneously into the right hind limb. FPL (100 mg/kg for 8 days) and/or CPA (25 mg/kg twice) were administered by intraperitoneal injection. Tumors were irradiated to a total dose of 40 Gy (eight 5-Gy fractions). SCCVII tumor growth was inhibited by FPL ($P = 0.054$), IR ($P = 0.003$) and CPA ($P < 0.001$) compared with control. The combination of FPL and CPA inhibited tumor growth additively compared with either treatment alone in both small- and large-volume tumors. FPL did not significantly enhance the antitumor effects of IR, however, when CPA + FPL were combined with IR, significant tumor growth inhibition was observed compared with FPL alone ($P < 0.001$), CPA alone ($P = 0.002$) and IR alone ($P = 0.002$). Due to its low toxicity profile, FPL may be combined with CPA, IR and other cytotoxic therapies to potentially enhance the therapeutic ratio.

Keywords Forphenicinol · Cyclophosphamide · Ionizing radiation · Tumor growth

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Introduction

Forphenicinol, L-2-(3-hydroxy-3-hydroxymethlyphenyl) glycine (FPL), is a low molecular weight immunomodifier synthesized in the early 1980s from forphenicine [1], an inhibitor of alkaline phosphatase [2]. Okura et al. [3] have demonstrated that oral administration of FPL restores delayed-type hypersensitivity in mice immunosuppressed by cyclophosphamide (CPA), and partially prevents mitomycin C-induced leukopenia. FPL has been reported to stimulate phagocytosis by activated macrophages in vitro and in vivo. Addition of FPL to bone marrow cultures has been shown to stimulate the growth of granulocyte-macrophage progenitor cells (CFU-C) [4] and increase the production of CFU-C in the presence of colony-stimulating factor [5]. Further immunomodulatory and antimicrobial effects of FPL have been reported in models of murine coccidiomycosis [6], in murine *Pseudomonas aeruginosa* infection [7], and in patients with mycobacterium [8].

The antitumor effects of FPL were first investigated by Ishizuka et al. In these studies, growth of both Ehrlich carcinoma and IMC carcinoma was suppressed by doses of FPL ranging from 0.31 to 5 mg/kg per day. FPL has also been employed in combination with various cytotoxic agents. When multiple injections of FPL (0.1–1 mg/kg per day) were administered with a single injection of CPA (50 mg/kg), enhanced antitumor effects were observed and survival time was prolonged in mice with L1210 leukemia. FPL (0.05–0.5 mg/kg per day) has also been shown to be effective when combined with

6-mercaptopurine (6-MP, 10 mg/kg per day) in suppressing the growth of sarcoma 180 (S180). However, weak antitumor activity was observed when FPL was used as a single agent to treat mice bearing Ehrlich carcinoma or S180 sarcoma [7]. Nitta et al. have demonstrated tumor growth inhibition following combined treatment with FPL and CPA in murine mammary carcinoma, L1210 leukemia, B16 melanoma, Lewis lung carcinoma (LLC) and glioblastoma tumors [9]. The enhancement of the antitumor effects of various antineoplastic agents by FPL may be due to restoration of the immune response, which is suppressed by cytotoxic agents [7]. The antitumor effects of FPL have also been examined following surgical resection of the primary tumor. Using the LLC and Meth A fibrosarcoma tumor models, Okura et al. have demonstrated that treatment with FPL suppresses the growth of recurrent tumors and prolongs survival [10].

The current studies are the first in which the effects of experimental treatment with FPL, CPA and ionizing radiation (IR) in a syngeneic model of squamous cell carcinoma have been investigated. We hypothesized that a strategy involving the use of a nontoxic compound such as FPL to enhance the efficacy of chemotherapy and radiotherapy may be useful in settings where standard treatments have proven ineffective. Our results demonstrate that FPL can be combined with cytotoxic therapies to achieve antitumor effects without increasing toxicity.

Methods

Tumor implantation

SCCVII murine squamous cell carcinoma cells, a gift from Ruth Modzelewski (University of Pittsburg, Pittsburg, Pa.), were maintained in RMPI 1640 (Invitrogen Life Technologies, Carlsbad, Calif.), 12.5% FBS (Intergen, Purchase, N.Y.), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Invitrogen Life Technologies). Female C3H/NEJ mice (Frederick Cancer Research Institute, Frederick, Md.) were injected subcutaneously (s.c.) into the right hind limb with 5×10^5 SCCVII cells in 100 μl PBS. Tumors were permitted to grow for 11–17 days prior to the initiation of treatment (day 0). Tumor volume was determined by direct measurement with calipers as described previously [11]. The initial tumor measurement represents the tumor volume on day 0, and based on these measurements, mice were randomly assigned to treatment groups such that the mean volume of each group was approximately equal [11–14].

Drugs

FPL was synthesized as described previously [1]. FPL was dissolved in PBS and injected intraperitoneally (i.p.) at doses of 5 mg/kg (nine injections, 45 mg/kg total dose) or 100 mg/kg (eight injections, 800 mg/kg total dose) depending upon the experiment. For all experiments, FPL was injected in a volume of 100 μl 1 h prior to IR exposure. CPA was purchased from the Bernard Mitchell Hospital pharmacy (University of Chicago), diluted in PBS and injected i.p. at doses of 50 mg/kg (five injections, 250 mg/kg total dose) or 25 mg/kg (two injections, total dose of 50 mg/kg). CPA was injected in a volume of 100 μl 3 h prior to IR exposure. Control mice and mice treated with IR alone were injected i.p. with a total of 200 μl PBS. Mice treated with FPL alone, or CPA alone, were injected with 100 μl PBS such that all experimental animals received a total i.p. injection volume of 200 μl.

Irradiation

Xenografts were irradiated using a Pantak PMC 100 X-ray generator at a dose rate of 1.92 Gy/min. SCCVII tumors were irradiated eight times using 5-Gy fractions, to a total dose of 40 Gy. This radiation dose was based on dose-finding studies conducted in our laboratory ($D_0 = 210$ cGy, data not shown). Mice were shielded with lead except for the tumor-bearing right hind limb [11]. The care and treatment of experimental animals was in accordance with institutional guidelines.

Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) and Student's *t*-test.

Results

SCCVII tumors are sensitive to cytotoxic therapy

To assess the individual effects of FPL, CPA and IR on the growth of SCCVII tumors, treatment with FPL or CPA was initiated on day 0 (mean tumor volume 278.1 ± 15.6 mm^3, $n = 32$) and IR treatment was begun on day 1. Control tumors grew exponentially reaching a mean volume of 4490 ± 454 mm^3 on day 14 (Fig. 1). Tumors in the FPL-alone group were 22% smaller (mean volume 3500 ± 123 mm^3, $P = 0.054$). Mice in these two treatment groups were killed due to tumor burden. Treatment with IR reduced mean tumor volume by 45% compared with control (2453 ± 355 mm^3, $P = 0.003$) while CPA produced a 97% reduction in mean tumor volume (124 ± 44 mm^3, $P < 0.001$). No systemic toxicity was detected following treatment with FPL, CPA or IR.

FPL interacts with CPA and IR to reduce mean tumor volume

We next investigated the effects of combined treatment with FPL, CPA and IR on SCCVII tumor growth. To evaluate potential interactions between cytotoxic agents

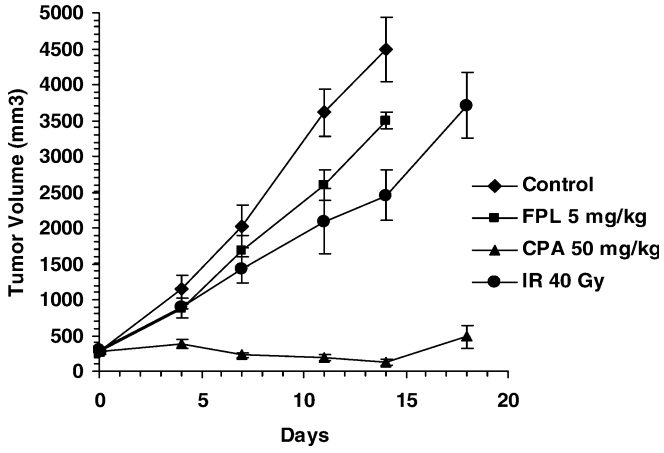

Fig. 1 Growth of SCCVII tumors following individual treatment with FPL, CPA or IR. Tumor-bearing C3H mice ($n=32$) were treated with FPL (5 mg/kg for 9 days), CPA (50 mg/kg for 5 days) or IR (5 Gy for 8 days). Mean tumor volume on day 0 was 278.1 ± 15.6 mm^3. SCCVII tumor growth was inhibited following treatment with FPL ($P=0.054$), IR ($P=0.003$) and CPA ($P<0.001$)

and based on the data from the previous experiment, the CPA dose was reduced, the FPL dose was increased (IR dose remained at 40 Gy). Mean tumor volume at the initiation of treatment (day 0) was 198 ± 5.6 mm^3 ($n=70$). On day 8 there was no difference in the growth of control tumors (2754 ± 360 mm^3) and tumors treated with FPL alone (2548 ± 120 mm^3; Fig. 2). Treatment with CPA alone reduced mean tumor volume by 54% compared with control. Tumors in the FPL + CPA group were 41% smaller (755 ± 208 mm^3) than tumors in the CPA-alone group (1276 ± 331 mm^3), supporting an interaction between CPA and FPL. Treatment with IR alone reduced mean tumor volume by 64% compared with control. The combination of FPL and IR produced a further reduction of 18% in mean tumor volume (FPL + IR 811 ± 192 mm^3, IR alone 993 ± 163 mm^3), also indicating interactive antitumor effects between FPL and IR. Triple therapy with FPL + CPA + IR produced a significant decrease (97%, $P<0.001$) in mean tumor volume compared with all other treatments (day 8). The suppression of tumor growth by triple therapy persisted until termination of the experiment on day 12.

Tumor size does not limit the antitumor effects of treatment with FPL, CPA and IR

We then examined the effects of treatment with FPL, CPA and IR on larger volume tumors (day-0 volume 426 ± 18.1 mm^3, $n=49$) using the same dose of each agent as described above. On day 11, tumors in the control group reached a mean volume of 3544 ± 386 mm^3. Treatment with CPA did not significantly slow tumor growth compared with control (mean volume 2848 ± 687 mm^3, Fig. 3). FPL enhanced the antitumor effects of CPA as evidenced by the fact that combined treatment with FPL and CPA significantly reduced mean tumor volume (1214 ± 161 mm^3) compared with CPA alone ($P=0.035$). Animals in the control, FPL and CPA groups were killed on day 11 due to tumor burden. A 57% reduction in mean tumor volume (1511 ± 202 mm^3) was observed following treatment with IR alone compared with control ($P<0.001$). Combined treatment with IR + FPL produced a further reduction of 13% (1311 ± 220 mm^3) compared with IR alone. Triple therapy (FPL + CPA + IR) produced a

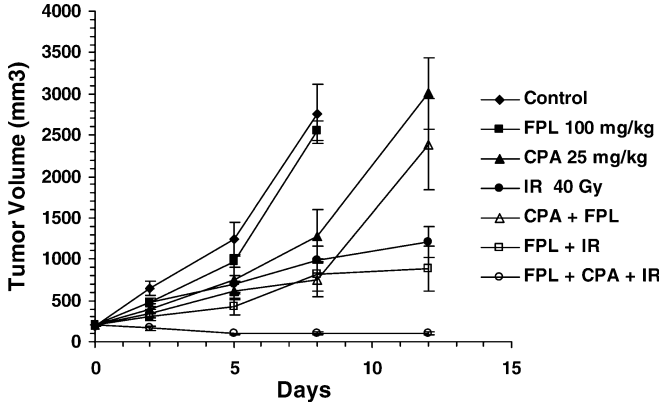

Fig. 2 Growth of small-volume SCCVII tumors exposed to FPL, CPA and IR as single agents and in combination. Tumor-bearing C3H mice ($n=70$) were treated with FPL (100 mg/kg for 8 days), CPA (25 mg/kg for 2 days) and IR (5 Gy for 8 days). Mean tumor volume on day 0 was 198 ± 5.64 mm^3. An interaction between FPL and CPA and between FPL and IR is apparent on day 8 following the initiation of treatment. Triple therapy with FPL + CPA + IR produced significant tumor growth inhibition ($P<0.001$)

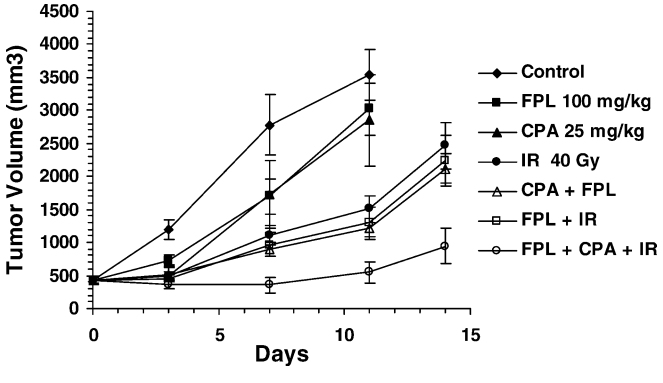

Fig. 3 Growth of large-volume SCCVII tumors exposed to FPL, CPA and IR. Mean tumor volume on day 0 was 426 ± 18.1 mm^3 ($n=49$). FPL enhanced the antitumor effects of CPA and IR. On day 11, triple therapy with FPL + CPA + IR had produced a significant reduction in mean tumor volume compared with control ($P<0.001$), FPL alone ($P<0.001$), CPA alone ($P=0.007$) and IR alone ($P=0.002$). On day 14, tumors in all treatment groups began to regrow. However, tumors exposed to triple therapy remained significantly smaller than tumors exposed to IR alone ($P=0.005$), FPL + CPA ($P=0.005$) or IR + FPL ($P=0.016$)

significant reduction in mean tumor volume compared with control ($P < 0.001$), FPL alone ($P < 0.001$), CPA alone ($P = 0.007$) and IR alone ($P = 0.002$). Between days 11 and 14, tumors in all surviving treatment groups (IR, FPL + IR and CPA + FPL) began to regrow. However, tumors exposed to triple therapy with FPL + CPA + IR remained significantly smaller ($P = 0.001$) than those exposed to IR alone ($P = 0.005$), FPL + CPA ($P = 0.005$) and IR + FPL ($P = 0.016$).

Discussion

The purpose of the present study was to evaluate the effects of FPL on combined chemotherapy and radiotherapy in a model of squamous cell carcinoma. We chose an immune-competent syngeneic model rather than an immune-suppressed xenograft model because FPL is reported to mediate antitumor effects through an enhanced immune response [3, 7, 10]. Murine SCCVII tumors were sensitive to the combined effects of FPL, CPA and IR treatment with no greater toxicity than with CPA and IR in combination. We conducted two experiments, one comprising small tumors, and a second with tumors greater than 400 mm^3 in volume. Although treatment with FPL alone had little effect on tumor growth, FPL enhanced the antitumor effects of CPA. Our findings are in agreement with previously published results [7, 9], but unlike those studies, in the current investigation treatment with FPL, CPA and/or IR was initiated after the primary tumor was established (11–17 days after tumor cell inoculation). Although we did not investigate the mechanism of interaction between FPL and CPA, it is possible that FPL exerts immunostimulatory effects as previously reported by others [5, 10]. In agreement with previously published studies, we detected no FPL-mediated toxicity even when the dose of FPL was escalated to six injections of 250 mg/kg each (data not shown). FPL has been used in clinical trials to determine an optimal dosing schedule for the treatment of cancer patients [15] and to evaluate the effects of FPL on the immune system [16]. FPL was found to increase both T cell and B cell populations with no adverse side effects with single oral doses ranging from 60 to 100 mg.

In our studies, FPL did not significantly enhance the antitumor effects of IR. These results may be related to the schedule of administration and/or the dose of FPL employed. CPA was injected prior to radiotherapy because this protocol has been reported to be the most effective [17]. Due to the sensitivity of SCCVII tumors, we injected CPA only two times for a total dose of 50 mg/kg in experiments 2 and 3. FPL was also injected prior to radiotherapy based on the findings of Ishizuka et al. [7], which suggest that FPL might restore the immune response which is damaged by cytotoxic agents such as CPA. Herein we report that when CPA and FPL were combined with IR, significant tumor growth inhibition was observed irrespective of tumor size. Our study is the first to show the effects of triple therapy with FPL, CPA and IR. A strategy involving the use of nontoxic compounds such as FPL to enhance the efficacy of chemotherapy and radiotherapy may be useful in settings where standard treatments have proven ineffective. Interactive treatment effects combined with a low toxicity profile suggest that FPL could potentially be combined with cytotoxic therapies to achieve antitumor effects.

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