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ORIGINAL ARTICLE

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Enhancing the therapeutic responsiveness of photodynamic therapy with the antiangiogenic agents SU5416 and SU6668 in murine nasopharyngeal carcinoma models

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Abstract *Background*: Photodynamic therapy (PDT) is a promising therapeutic modality using a tumor localizing photosensitizer and light to destroy tumor cells. A major limitation of PDT is tumor recurrence, which is partly due to neovascularization. *Purpose*: The objective of the present study was to determine whether combination therapy with PDT and antiangiogenic agents (i.e. SU5416 and SU6668) would be more effective in controlling tumor recurrence in a mouse model of human CNE2 poorly differentiated nasopharyngeal carcinoma compared with PDT or antiangiogenic agents administered alone. *Methods*: Athymic mice bearing CNE2 tumor xenografts received daily i.p. injections of 20 mg/kg SU5416 or 100 mg/kg SU6668 for 28 consecutive days either alone or following a single hypericin-PDT treatment. *Results*: Significant inhibition of CNE2 tumor growth was observed in all treatment groups. Differences in 4× tumor growth time, the number of mice with 4× tumor growth, tumor growth inhibition as well as the percent of mice surviving were not statistically significant among individual treatment groups. However, the number of mice with 4× tumor growth observed in SU6668 monotherapy and combined PDT and SU6668 treatment groups was significantly less than that in the control group ($P < 0.05$ and 0.01, respectively). Moreover, compared with the control group, only the combined PDT and SU6668 treatment significantly extended survival of tumor-bearing host mice ($P < 0.05$). The semiquantitative RT-PCR results showed that the expression of HIF-1α, VEGF, COX-2 and bFGF were increased in PDT-treated tumor samples collected 24 h post-PDT, suggesting that PDT-induced damage to tumor microvasculature and the resultant hypoxia upregulate the expression of certain proangiogenic factors. *Conclusions*: The effectiveness of PDT can be enhanced by antiangiogenic treatment with the synthetic RTK inhibitors. Of the two synthetic RTK inhibitors tested, SU6668 was more effective than SU5416 in enhancing tumor responsiveness to PDT.

Keywords Photodynamic therapy · Antiangiogenic agents · Nasopharyngeal carcinoma

Abbreviations bFGF: Basic fibroblast growth factor · CCE: Choriocapillaris endothelial · COX-2: Cyclooxygenase-2 · FGFR1: Basic fibroblast growth factor receptor-1 · GAPDH: Glyceraldehyde-3-phosphate dehydrogenase · HIF-1: Hypoxia-inducible factor 1 · i.p.: Intraperitoneal · NPC: Nasopharyngeal carcinoma · PDGF: Platelet derived growth factor · *PDGFR*: Platelet derived growth factor receptor · PDT: Photodynamic therapy · RTK: Receptor-associated tyrosine kinase · TK: Tyrosine kinase · VEGF: Vascular endothelial growth factor · VEGFR-2: Vascular endothelial growth factor receptor 2

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Introduction

Tumorigenesis involves a process involving multiple molecular and cellular events [34, 37]. Advances in cancer treatment, with surgery, radiotherapy and conventional

cytotoxic chemotherapy, have hitherto made only a modest impact on mortality. Clinical needs for innovative therapeutic strategies to achieve better cancer treatment still remain. Photodynamic therapy (PDT) is a relatively new treatment modality involving the administration of a photosensitizing agent and its subsequent activation by light in the presence of molecular oxygen to produce a highly toxic singlet oxygen to kill tumor cells [9]. The appeal of PDT in cancer treatment is that the photosensitizer tends to be retained in tumor tissues for a longer period of time as compared with normal tissues, resulting in a large therapeutic index [17]. Furthermore, the use of PDT is not precluded by prior radiotherapy, chemotherapy or surgery [9]. The effect of PDT on the destruction of tumor tissues has been known to be attributed to both direct cytotoxic activity and microvascular damage [15]. The vascular effects of PDT involve destruction of tumor blood vessels as evidenced by vascular thrombosis and leakage resulting in tissue oedema, blood flow stasis, hemorrhage and necrosis [15, 25, 33]. The mechanisms underlying the vascular effect of PDT differ greatly with different photosensitizers [2]. Hypericin-PDT, for example, induces severe damage of tumor vascular endothelial cells, resulting in the collapse of the entire microcirculation system [3].

A major limitation of PDT, however, is tumor recurrence [31]. Although exposure of tumors to PDT can reduce the number of clonogenic tumor cells through direct photodamage, this alone may be insufficient for tumor cure. Furthermore, the success of PDT also depends on the stage of disease and tumor size. Studies in rodent systems employing curative procedures with several photosensitizers showed direct photodynamic tumor cell kill rate to be less than 2 logs and in most cases, less than 1 log, which is far short of the 6–8-log reduction required for tumor cure [1, 12, 14]. Two mechanisms can account for the incomplete tumor kill: (a) the photochemical consumption of oxygen during the photodynamic process, and (b) effects of PDT on the tissue microvasculature. Following PDT, a state of hypoxia is induced within the tumor tissue as a result of rapid oxygen consumption [4, 11, 28]. This hypoxic state mediates several adaptive gene expressions through the transcription factor hypoxia-inducible factor 1 (HIF-1); activation occurs through the oxygen-regulated stabilization of HIF-1α followed by dimerization with HIF-1β [6, 29, 38]. VEGF expression, for example, has been shown to be increased in hypoxic tumor tissues as a result of both transcriptional activation and increased stabilization of HIF-1α [21]. PDT-induced damage to tumor microvasculature and the resultant hypoxia in the tumor may stabilize HIF-1α, leading to upregulation of VEGF, and possibly other pro-angiogenic growth factors, which can then promote angiogenesis [11].

Since tumor recurrence due to neovascularization can occur after PDT, methods to block angiogenesis may have implications in improving the therapeutic responsiveness of solid tumors to PDT. The enhancement of PDT tumor responsiveness by antiangiogenic treatments targeting VEGF or other angiogenic growth factor receptor-associated tyrosine kinases (RTKs) in experimental tumor models, has been documented [7, 11]. SU5416 is a potent and competitive inhibitor of VEGF receptor-2 with respect to adenosine triphosphate (ATP) with an inhibition constant (K_i) value of 0.16 μM [13, 23]. Although SU5416 has been shown to inhibit VEGF-dependent endothelial cell proliferation in vitro and in animal models [13, 36], in recent phase II studies, SU5416 monotherapy only exhibited modest clinical activities in patients with different types of solid tumors, including melanoma, myeloma and prostate cancer [27, 32, 40]. Compared with SU5416, SU6668 is a relatively broad spectrum RTK inhibitor for VEGF, FGF and PDGF receptors [16, 18]. SU6668 has been shown to have competitive inhibitory properties with respect to ATP to inhibit phosphotyrosine levels in VEGF receptor-2 and platelet-derived growth factor receptor β in vivo, resulting in rapid tumor vasculature apoptosis followed by apoptosis of tumor cells [19]. Moreover, SU6668 treatment has been shown to result in regression or marked growth inhibition of many large established human tumor xenografts in mice [18]. An early in vivo study has also demonstrated the abilities of SU5416 and SU6668 to potentiate the antitumor effect of fractionated irradiation in mice [26]. In this study, we aimed to determine whether combination therapy with PDT and antiangiogenic agents (i.e. SU5416 and SU6668) would be more effective in controlling the recurrence of CNE2 tumor xenografts in athymic mice as compared with PDT or antiangiogenic agents administered alone. Since the expression of angiogenic growth factors may vary at different stages of tumor development, effects of these antiangiogenic agents post-PDT were evaluated at different stages of the CNE2 tumor growth.

Materials and methods

Chemicals

SU5416 and SU6668 were kindly supplied by Sugen, Inc. (South San Francisco, CA, USA). Hypericin was purchased from Molecular Probes (Eugene, OR, USA). Dimethyl sulfoxide (DMSO) was of HPLC grade and obtained from Sigma Co. (St. Louis, MO, USA). SU5416 and SU6668 were dissolved in DMSO and injected intraperitoneally (i.p.) with a dose volume of 10 mL/kg at daily doses of 20 and 100 mg/kg, respectively, per animal for 28 consecutive days. Hypericin (0.4 mg/ml) was freshly prepared in 40% DMSO in phosphate-buffered saline (PBS) before injected to the mice.

Animals

Male BALB/c athymic (nu+/nu+) mice were purchased from the Animal Resources Center (Murdoch, Australia) and used for xenografting at the age of

8–10 weeks. Animal experiments were approved by the institutional ethics committee for animal research in the National Cancer Center (Singapore). Animals were housed in air-filtered laminar flow cabinets in microisolator cages under pathogen-free conditions and kept on a 12-h light/dark cycle for 1 week before receiving injections of cancer cells. Autoclaved standard chow and water were available ad libitum.

Cell Culture

Poorly differentiated human nasopharyngeal carcinoma cell line (CNE2) was a gift from Prof. Hui Kam, Man of the National Cancer Center (Singapore). CNE2 tumor cells were maintained in RPMI 1640 medium containing 10% standard fetal bovine serum (FBS, Hyclone, USA), 1 mM MEM sodium pyruvate solution, 0.1 mM non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, USA), in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Xenografts

CNE2 tumor cells at logarithmic growth in vitro were harvested and washed twice with phosphate-buffered saline (PBS). The tumor cells (1×10^6) in a form of pellet after centrifugation at low speed (1,000×*g*), were re-suspended in 100 μl Hank's buffer (Gibco BRL, Invitrogen Co., Carlsbad, CA, USA) and inoculated subcutaneously in the right flanks of the athymic mice through a 22-gauge needle. Tumor growth was monitored twice a week, and mice were randomized into drug-treated and control groups (11∼13 mice/group) when the tumor volume reached approximately 0.2 cm^3. Two perpendicular diameters (d_1 and d_2) were measured with a vernier caliper (Scienceware, Bel-Art, NJ, USA), and tumor volumes (V) were calculated using the following formula [22]:

$$V = 0.35 \times (d_1 \times d_2)^{3/2} \quad (1)$$

In vivo treatment protocol

Six groups of male athymic mice ($n = 11$–13 per group), in a parallel design, were given the following treatments: group A: vehicle control; group B: PDT treatment alone; group C: SU5416 treatment alone; group D: SU6668 treatment alone; group E: PDT followed by SU5416; group F: PDT followed by SU6668 (Fig. 1). Groups C–F were treated with either SU5416 20 mg/kg/d i.p. or SU6668 100 mg/kg/d i.p., while vehicle control animals only received DMSO 10 ml/kg/d i.p. for 28 consecutive days. Athymic mice receiving PDT were given a single intravenous (i.v.) dose of hypericin (2 mg/kg) on day zero. Two hours later, the hypericin-treated mice were photoirradiated with a halogen light source that was fitted with a red acetate filter (No. 17, Roscolux, Rosco, Markham, Canada). Tumors were photoirradiated with 47.7 J/cm^2 at a fluence rate of 60 mW/cm^2. Both SU5416 and SU6668 treatments were started on day 1, irregardless of whether the mice were given a single hypercin-PDT treatment on day zero. On days 1, 14 and 28 (groups A and B) or days 14 and 28 post-PDT (groups C–F) some mice were euthanized with CO_2 and the tumor mass was excised. The tumor was dissected using a razor blade and snap frozen in liquid nitrogen and then stored at −80°C before RNA isolation. Other animals were followed up for 42 days after completion of the treatment period (Fig. 1).

Body weights and tumor volumes were determined twice a week throughout the experiment. The effectiveness of individual treatments were evaluated by the tumor growth inhibition, 4× tumor growth time, the number of mice with 4× tumor growth during the treatment period and the percent of mice surviving. The tumor growth inhibition of each treatment group was calculated according to the following formula: % growth inhibition = [(mean TV_c - mean TV_t)/mean TV_c] × 100%, where mean TV_t is the average tumor volume of treated tumors while mean TV_c is that of vehicle control tumors measured on the same treatment day. Quadrupling (4×) tumor growth time is the time in days

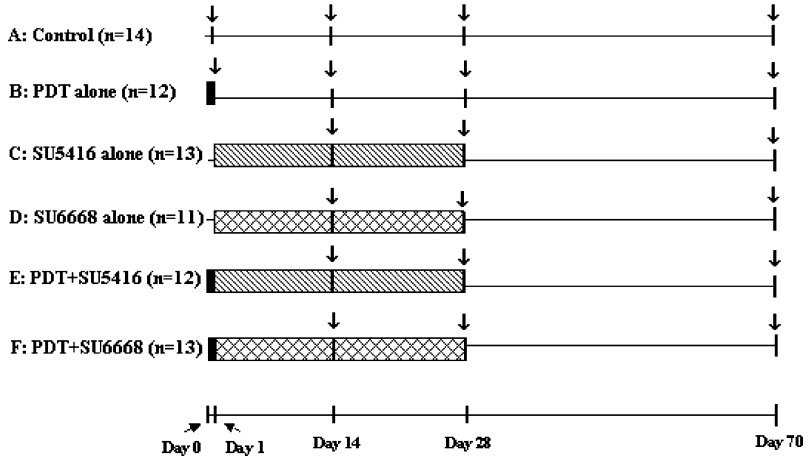

Fig. 1 Diagrammatic representation of PDT, SU5416 and SU6668 monotherapy as well as PDT and SU5416 combined-therapy and PDT and SU6668 combined-therapy procedures in nude mice bearing a CNE2 xenograft. PDT (hypericin 2 mg/kg, i.v.); SU5416 treatment (20 mg/kg/d, i.p.); SU6668 treatment (100 mg/kg/d, i.p.); ↓ Being sacrificed

required for individual tumor to reach a fourfold increase in volume.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from dissected tumor tissues using TRIzol Reagent (Invitrogen Co. Carlsbad, CA, USA) according to the manufacturer's instructions, and quantified by spectrophotometry. The quality of total RNA was examined by visualizing the RNA electrophoresis on a denaturing agarose gel. The presence of intact bands of 28s and 18s ribosomal RNA should be inspected and the ratio 28s/18s should be more than 1.

First-strand cDNA was synthesized by reverse transcription (RT) of 2 μg total RNA with the following reagents in a 20 μl reaction mixture: 0.5 μg of oligo(dT)15 primer, 4.0 μl of ImProm-II 5× reaction buffer, 3.0 mM $MgCl_2$, 500 μM each of dNTP, 20 U of ribonuclease, and 1 μl ImpromII reverse transcriptase (Promega Co., Madison, WI, USA). Reactions were carried out in the MJ Research PTC-200 thermocycler (MJ Research Inc., Watertown, MA, USA) with one cycle of the RT reaction (25°C for 5 min, 42°C for 60 min, and 70°C for 15 min). Negative controls containing either no sample RNA or no ImpromII^{TM} Rtase were included. The resulting cDNA was stored at −20°C.

The primer sequences for *GAPDH*, *VEGF, HIF-1α*, *COX2*, *bFGF*, and *PDGFβ*, respectively, were created based on the published mRNA sequences and are listed in Table 1. PCR amplifications were carried out with the following reagents in a 20-μL reaction mixture: 0.5 μl of cDNA, 0.25 μM of each primer, 10×PCR buffer containing 10 mM Tris and 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTP and 1 U *Taq* DNA polymerase. PCR conditions consisted of an initial denaturation at 94°C for 2 min, followed by amplification using 35 cycles of 94°C for 30 s, 55°C for 30 s, and elongation at 72°C for 1 min, followed by a final extension for 7 min at 72°C. Aliquots (10 μl) of the amplification products were separated by electrophoresis through a 2.0% agarose gel and visualized by ethidium bromide staining. The intensity of each band was quantified by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Only RNA samples that gave completely negative results in PCR without reverse transcriptase were used to rule out the presence of genomic DNA contamination. In this study, the housekeeping gene *GAPDH* was used as an internal standard. The expression of each targeted gene was normalized to the expression of *GAPDH* and expressed as the ratio of band intensities of the particular gene to *GAPDH*.

Statistical analysis

Data analyses were performed using STATA (StataCorp, 2001. STATA Statistical Software: Release 7.0. Stata Corporation, College Station, TX, USA). Sample data were expressed as mean ± standard deviation (SD). Comparisons of means of related samples were made using two-sided paired *t*-test. In case of multiple comparisons, one-way ANOVA followed by the post hoc multiple comparisons with Tukey's honestly significant difference test, were used. Fisher's exact test was performed for the comparison of the number of mice with tumor volume less than 4 times of the initial volume throughout the monitoring period in individual groups. Log-rank test for the equality of survivor functions was carried out for the survival analysis. A *P*-value of less than 0.05 was considered statistically significant.

Results

Body weight changes during treatment

During the treatment period, the body weight loss for all groups never exceeded 16%. One-way ANOVA results showed lack of statistically significant differences in body weight between the control and individual treatment groups prior to and during the period of treatment ($P > 0.05$).

Table 1 Summary of PCR primer sequences

Gene	Primer	Sequence	PCR product size (bp)	Genbank accession no.
GAPDH	GAPDHF	5′-GGAAGGTGAAGGTCGGAGTC-3′	556	M33197
	GAPDHR	5′-GTCTTCTGGGTGGCAGTGAT-3′		
HIF-1α	HIFF	5′-TTCTGGATGCTGGTGATTTG-3′	450	U22431
	HIFR	5′-GCACCAAGCAGGTCATAGGT-3′		
COX2	COX2F	5′-CCGGACAGGATTCTATGGAGA-3′	300	M90100
	COX2R	5′-CAATCATCAGGCACAGGAGG-3′		
VEGF	VEGFF	5′-TCCAGGAGTACCCTGATGAG-3′	386 and 452	AF430806 and M32977
	VEGFR	5′-CTTTCCTGGTGAGAGATCTGG-3′		
bFGF	bFGFF	5′-CCATCCTTTCTCCCTCGTTT-3′	141	NM_002006
	bFGFR	5′-TCCCTCCAATGTTTCATTCA-3′		
PDGFβ	PDGFbF	5′-TCCCGAGGAGCTTTATGAGA-3′	458	NM_033016
	PDGFbR	5′-ATGCCAGGTGGTCTTCCA-3′		

Inhibition of growth of CNE2 tumor xenografts in athymic mice

The antitumor effects of different treatments were evaluated based on the inhibition of tumor growth and the results are shown in Table 2 and Fig. 2. As shown in Fig 2, tumor growth was significantly suppressed in mice receiving different treatments compared with the control group, but to varying degrees: 53% for group B; 54% for group C; 80% for group D; 80% for group E; 84% for group F. Athymic mice receiving either SU5416 or SU6668 monotherapy had significantly longer 4× tumor growth time compared with the control animals ($P < 0.05$ for both). Four of nine mice in group D and one of nine mice in group F developed 4× tumor growth during the treatment period, which were significantly different from control mice (group A) by day 24 ($P < 0.05$ and 0.01, respectively). Comparison of differences in tumor growth among different treatment groups showed that treatment with SU6668 (group D) significantly inhibited tumor growth as compared with PDT alone (group B) on day 21 (80% vs 20%, $P < 0.05$). Moreover, significant differences in tumor growth were found between groups B and *F* on day 7 ($P < 0.01$), and between groups D and *F* on day 14 ($P < 0.05$), suggesting that PDT followed by daily administration of SU6668 tends to be more effective than either treatment given alone. After the termination of treatment with SU5416 or SU6668, tumors grew rapidly in most of the treatment groups except group E, in which the growth delay in established tumors persisted for up to 6 weeks. It was noted that the tumor volume tended to decrease in groups B, C and E at later times (Fig. 2). This was because certain animals with larger tumors died earlier, leaving those with smaller tumors for measurement.

Table 2 Antitumor effect of treatments CNE2 cells were inoculated subcutaneously in the right flank of the athymic mice. Treatment was initiated when xenografts reached about 0.2 cm^3. Treatment groups received hypericin (2 mg/kg) by intravenous administration, and received SU5416 (20 mg/kg/day) or SU6668 (100 mg/kg/day) by intraperitoneal administration

Group	Number of mice with tumor volume <4 times of the initial volume throughout the monitoring period	4×tumor growth time (±SD)[b] (days)
Group A	0 (9)[a]	9 ± 5
Group B	1 (7)	16 ± 5
Group C	2 (9)	20 ± 8***
Group D	5 (9)*	21 ± 8***
Group E	4 (7)	21 ± 12
Group F	8 (9)**	21

[a] Figures in parentheses indicate the number of athymic mice monitored

[b] Four times of the initial tumor volume measured on day 0

* $P < 0.05$, ** $P < 0.01$ compared with the control groups using Fisher's exact test; *** $P < 0.05$ compared with the control group using one-way ANOVA

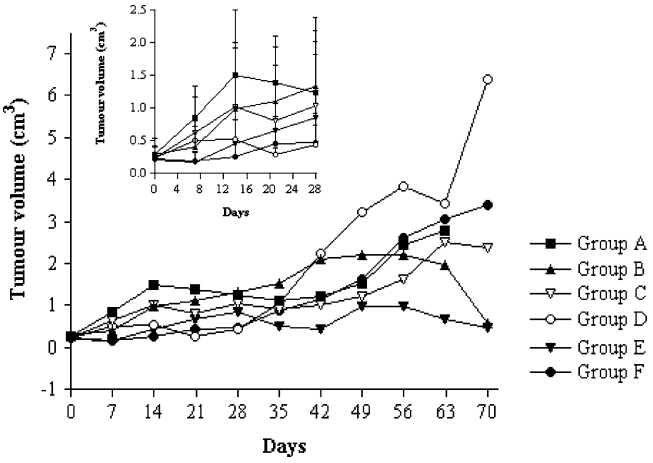

Fig. 2 The effect of individual treatment on tumor growth in athymic nude mice bearing established CNE2 xenografts. CNE cells (1×10^6) were injected s.c. and treatment was initiated when xenografts reached about 0.2 cm^3. Mice bearing CNE2 xenografts were treated with vehicle (group A), PDT with 2 mg/kg hypericin (group B), 28-day i.p. injection of 20 mg/kg SU5416 alone (group C), 28-day i.p. injection of 100 mg/kg SU6668 alone (group D), PDT followed by 28-day i.p. administration of 20 mg/kg SU5416 (group E), and PDT followed by 28-day i.p. administration of 100 mg/kg SU6668 (group F)

Animal survival

Fig. 3 shows Kaplan-Meier survival curves for different treatment groups. The percent of surviving mice values were 0%, 33%, 75%, 33%, 33% and 100% for groups A, B, C, D, E and F, respectively. A significant prolongation in survival was only observed in mice treated with PDT followed by SU6668 administration (Group F) ($P < 0.05$).

Effect of SU5416 and SU6668 on *VEGF*, *COX2*, *HIF-1α*, *bFGF* and *PDGFβ* expression levels in CNE2 xenografts

The expression levels of *VEGF, HIF-1α, COX2, bFGF* in PDT-treated tumor samples collected 24 h after PDT were higher than those in control samples (126%, 303%, 153% and 210% of control, respectively; $P > 0.05$ for all) (Fig. 4a–e). The expression of *PDGF* was not detected in the PDT-treated tumor samples. By day 14, the expression levels of *VEGF*, *COX-2*, *HIF-1α* and *bFGF* in most of the treatment groups appeared to be relatively low compared with those in the control group. By day 28, most of the treatment groups were found to have almost the same or higher expression levels of *VEGF*, *COX-2*, *HIF-1α*, *bFGF* and *PDGFβ* compared with the control group (Fig. 4a–e). Nonetheless, it was noted that expression levels of *HIF-1α* and *bFGF* were relatively lower in mice receiving treatment with SU6668 compared with those receiving SU5416 regardless of whether PDT was administered prior to the antiangiogenic treatments (Fig. 4c, 4d). Since all the mice in the control

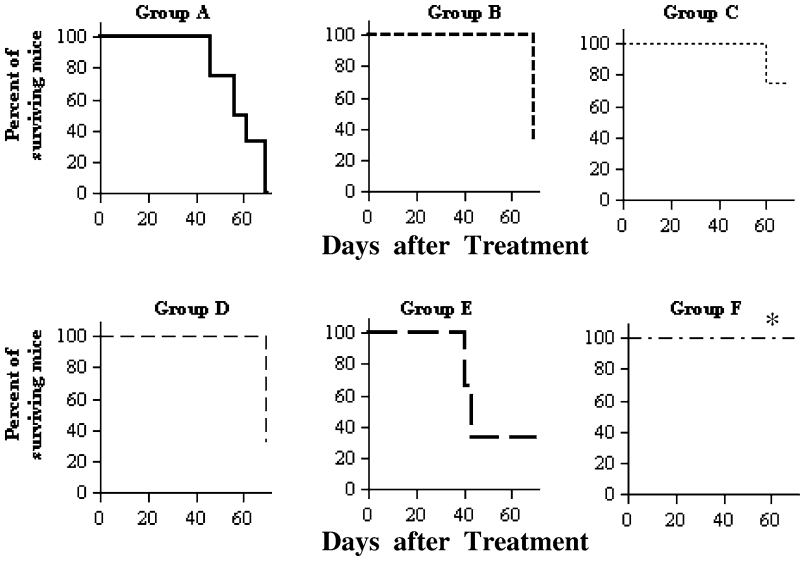

Fig. 3 Kaplan-Meier plot of the survival of animals receiving vehicle, or PDT once, or 28-day administration of SU5416 (20 mg/kg/day) or SU6668 alone (100 mg/kg/day), or 28-day administration of SU5416 or SU6668 given 24 h after PDT. Monitoring period was 70 days. The number of mice monitored was: group A, 4; group B, 3; group C, 4; group D, 3; group E, 3; and group F, 3. Animal survival was noted on a daily basis. Statistical analyses were performed using the log-rank test. * $P < 0.05$ compared with the control group

group died during the follow-up period, no tumor tissue was available for total RNA quantification. Also, the total RNA could not be isolated from the necrotic tumor tissue collected from the only surviving mouse in group B at the end of the follow-up. For the rest of the treatment groups, the mean expression levels of *VEGF, COX-2* and *HIF-1α* appeared to be relatively low in mice treated with SU5416 either alone or following PDT as compared with those in SU6668-treated mice regardless of prior PDT treatment (Fig. 4a–c). However, the results of one-way ANOVA showed no statistically significant difference in *VEGF, COX-2*, *HIF-1α, bFGF* and *PDGF*β levels among different groups on days 14, 28 and 70 ($P > 0.05$).

Discussion

The PDT continues to be an important treatment modality for a variety of malignant and non-malignant tumors [8, 9]. Over the last decade, the effectiveness of PDT in the treatment of NPC, one of the most common malignancies in southern China and Southeast Asia [39], has been evaluated in both experimental and clinical studies [10, 20, 35]. It has been suggested that PDT, which carries no serious side effects, can be used for recurrent or persistent NPC in cases where very high doses of ionizing radiation have failed [20, 35]. However, the main limiting factor in PDT is tumor recurrence and several aspects may contribute to this phenomenon. The tumor type and its sensitivity to the photosensitizers used, the irregular distribution of photosensitizers and light source within the tumor and the buildup of hypoxic environment intratumorally following PDT may all compromise the therapeutic effectiveness of PDT [31]. The rationale was that an antiangiogenic agent may prevent or delay post-PDT tumor recurrence by inhibiting PDT-induced elevation of proangiogenic factors.

In this study, no significant body weight loss and changes of grooming habits were observed in athymic mice treated with SU5416 or SU6668, demonstrating that SU5416 and SU6668 are well tolerated with favorable toxicity profile when administered at 20 mg/kg and 100 mg/kg, respectively, on a daily basis.

Both SU5416 and SU6668 have been reported to inhibit the formation of metastases and microvessel formation and increase apoptosis of both tumor endothelial cells and tumor cells in mouse xenograft models [30]. In the present study, both SU5416 and SU6668 inhibited CNE2 tumor growth after once-a-day dosing, either alone or following PDT. Tumor growth was inhibited to a greater extent in mice treated with SU6668 compared with SU5416. Moreover, a statistically significant decrease in the number of mice with 4× tumor growth was only found in SU6668-treated mice during the treatment period (Table 2). These observations suggest that SU6668 is more effective than SU5416 in inhibiting PDT-induced tumor regrowth. It is possible that the broad spectrum RTK inhibitor, SU6668, through its blockade of multiple receptor target sites, is more potent in inhibiting angiogenesis and hence tumor regrowth, compared with the selective RTK inhibitor, SU5416. However, once the antiangiogenic treatment ended, tumor volumes increased markedly in SU6668-treated mice compared with those in SU5416-treated mice, suggesting a relatively longer duration of blockade due to SU5416. This observation was in accordance with a previous in vitro study, which showed that a short-term exposure to SU5416 produced a long-lasting inhibition of the VEGF-dependent proliferation in cells, while SU6668 did not demonstrate such long-lasting activity in similar studies [24]. One possible reason for this observation is that the highly hydrophobic nature of SU5416 (Log $D > 5$) compared with SU6668 makes it prone to bind to the lipid membrane, and subsequently, to be released gradually into the cytosolic fraction of the

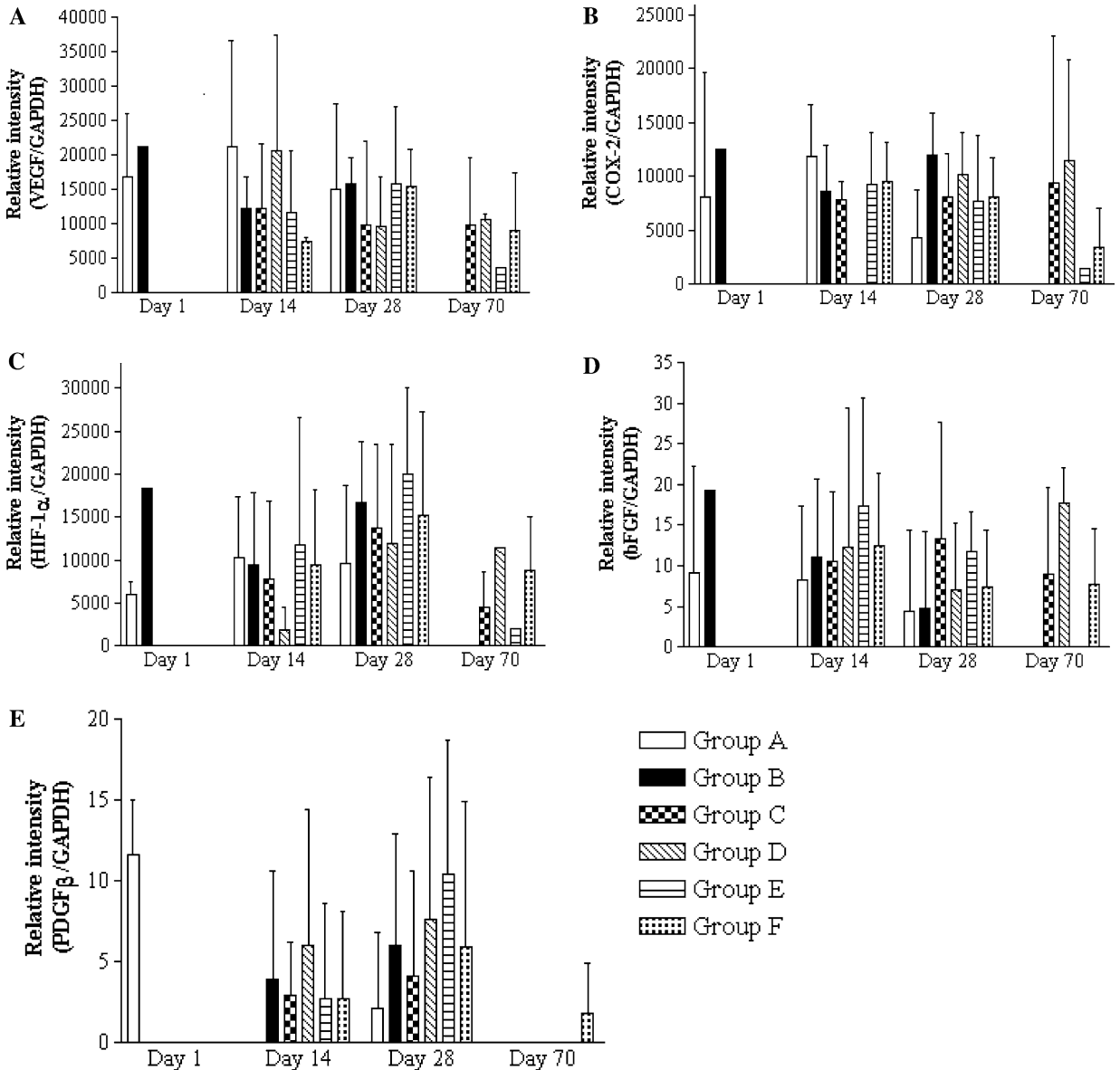

Fig. 4 Expression of VEGF (**A**), COX-2 (**B**), HIF-1α (**C**), bFGF (**D**), and PDGFβ (**E**) in the tumors harvested from the tumor-bearing mice in groups A and B killed on days 1, 14, 28 and 70, and in groups C, D, E and F on days 14, 28 and 70 after the initiation of the treatment. GAPDH was used as internal control. Results are presented as the ratio of band intensities of the particular gene to GAPDH. *Vertical bars* SD

cell to maintain the inhibitory concentration at the VEGF receptor site. Another possibility is that SU5416 has been shown to be highly specific for VEGF receptor-2 (VEGFR-2) with an IC_{50} of 1.0 μM in biochemical assays [13], whereas SU6668 was less specific, with an IC_{50} of 2.1 μM for VEGFR-2 [18]. In this regard, the longer duration of the inhibitory effect of SU5416 on tumor growth may be attributed to its greater potency of blocking VEGFR-2 compared with SU6668 because the VEGF family and their corresponding tyrosine kinase receptors are known to play a central role in the angiogenic process, and VEGFR-2 is the major receptor transducing the mitogenic and survival signals of VEGF into endothelial cells in tumor blood vessels [5].

Tumor growth in mice receiving PDT alone was inhibited significantly compared with the untreated mice, but to a lesser extent in comparison with mice receiving either PDT followed by treatment with the antiangiogenic agent (i.e. SU5416 or SU6668) or monotherapy with the antiangiogenic agent. PDT-induced hypoxia can promote the upregulation of angiogenic and survival pathways in tumors, and subsequently result in tumor resistance to PDT. As shown by RT-PCR, the expression levels of HIF-1α, VEGF, FGF and COX-2 after PDT were relatively high compared with controls, suggesting that the compromised inhibitory effect of PDT on tumor growth is related to the increased expressions of growth factor due to a

PDT-induced hypoxic state (Fig. 4). No significant differences in the expression levels of HIF-1α, COX-2, VEGF, FGF and PDGFβ were found among the groups of mice receiving either post-PDT treatment or monotherapy with SU5416 or SU6668, suggesting that the expressions of those pro-angiogenic factors are modulated by SU5416 and SU6668 to a similar degree. Similar results were also reported in other studies [7, 11]. In the study by Ferrario et al. [11], the potentiation of responsiveness to PDT by antiangiogenic agents were dramatic compared to the present study. One possible reason could be that the PDT dose delivered in this study was much lower compared with that used in the study by Ferrario et al. [11].

Although our data showed that SU5416 had a relatively lasting inhibitory effect on tumor growth, it was noteworthy that mice given SU6668 post-PDT survived longer periods as compared with any other treatment groups ($P < 0.05$; Fig 3). This observation suggests that inhibition of multiple tumor signal transduction pathways critical to tumor growth and survival is more effective than inhibition of one key regulator of angiogenesis. Taken together, the results of the present study demonstrated that the therapeutic efficacy of PDT can be enhanced with the addition of antiangiogenic agents. The effect of the broad spectrum RTK inhibitor SU6668 on delaying CNE2 tumor growth was superior to that of the narrow spectrum RTK inhibitor SU5416. Moreover, since the present study has shown that combining PDT with antiangiogenic therapy is effective against CNE2 tumor xenografts, and NPC is more prevalent in the eastern part of the world, the effectiveness of this combined treatment modality should be tested in a clinical trial setting.

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