

Efficacy of BIBF 1120 or BIBF 1120 plus chemotherapy on nasopharyngeal carcinoma in vitro and in vivo

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Purpose: BIBF 1120 is a potent triple angiokinase inhibitor now being evaluated in many types of tumors. We examine the antitumor effects of BIBF 1120 on nasopharyngeal carcinoma (NPC) in vitro and in vivo.

Materials and methods: The effect of BIBF 1120 on NPC cell proliferation was evaluated using the Cell Counting Kit 8 assay. The activities of BIBF 1120 as a single agent and in combination with cisplatin (DDP) in NPC tumor xenografts were evaluated by measuring microvessel density and expression of vascular endothelial growth factor signaling.

Results: BIBF 1120 exhibited limited inhibition of the growth of three NPC cell lines. Concurrent administration of BIBF 1120 and DDP provided greater antitumor effects compared to that observed with the use of either inhibitor as a single agent in the NPC xenograft model. Microvessel density and expression of vascular endothelial growth factor signaling were significantly reduced.

Conclusion: BIBF 1120, either as a single agent or in combination with DDP, demonstrates significant antitumor and antiangiogenic effects in the NPC xenograft model. Our results indicate that BIBF 1120 administered in conjunction with chemotherapy might provide an effective treatment method for NPC.

Keywords: BIBF 1120, nasopharyngeal carcinoma, antiangiogenesis, microvessel density

Introduction

Nasopharyngeal carcinoma (NPC) is the most common head and neck cancer in Southern China, where its incidence rate ranks the highest worldwide (20–30 per 100,000).^{1–3} In spite of the relatively high response rates to chemotherapy (~60%)^{4,5} and emerging new drugs, survival in advanced disease cases is poor (progression-free survival of 5–10 months and overall survival [OS] of 7–19 months).^{6–8} It is imperative to explore novel treatments, especially targeted therapies, for patients with advanced NPC.

The vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) system is a potential target for antiangiogenic therapy as angiogenesis constitutes an important pathway for NPC progression and metastasis. Overexpression of VEGF/VEGFR is observed in most patients with NPC and is related to an increased risk of metastasis and shortened OS,^{9–13} providing a rationale for investigating antiangiogenic agents for NPC.

A number of antiangiogenic inhibitors have been studied in clinical settings. However, acquired resistance was commonly observed in anti-VEGF/VEGFR treatments.^{14,15} Previous studies have revealed that the inhibition of the VEGF/VEGFR pathway results in the activation of alternative pathways to maintain neo-angiogenesis in the tumor. Fibroblast growth factor (FGF)/FGF receptor and platelet-derived growth

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factor (PDGF)/PDGF receptor pathways are acknowledged as the main pathways involved in the potential alternative mechanisms.¹⁶ Fernando et al¹⁷ found that tumor cells could escape angiogenesis inhibition by upregulating various pro-angiogenic factors, including FGF and PDGF. Multiple blockage of these escape mechanisms might delay the revascularization of the tumor and its progression.¹⁶

BIBF 1120, a structurally optimized oxindole derivative, competitively inhibits ATP binding to VEGFR2 and other angiogenic receptor tyrosine kinases. BIBF 1120 powerfully blocks VEGFR, PDGF receptor, and FGF receptor kinase activity in enzymatic assays (IC_{50} [half maximal inhibitory concentration] values were 13 nM, 59 nM, and 37 nM, respectively). Moreover, BIBF 1120 has been shown to inhibit the growth of various human tumor xenografts and prevent tumor angiogenesis in vivo in a dose-dependent manner. Pharmacokinetic studies in mice showed the highest and lowest plasma concentrations, of ~1,000 nmol/L and 8 nmol/L, at 1 hour and 24 hours postadministration, respectively.^{18–20} Results of a Phase III randomized clinical trial, Lume-Lung 1, revealed that progression-free survival of patients with non-small cell lung cancer who failed previous chemotherapy, when treated with docetaxel plus BIBF 1120, was significantly longer than that of patients treated with docetaxel alone (3.4 vs 2.7 months, $P=0.0019$). OS was improved from 6.3 months to 9.8 months in patients with adenocarcinoma. Due to its efficacy, BIBF 1120 plus docetaxel has been currently approved as a second-line treatment for advanced lung adenocarcinoma in Europe.²¹

Based on its novel characteristics, we assumed that BIBF 1120 would exhibit antiangiogenic activity in preclinical models of NPC. As cisplatin (DDP) is the foundational chemotherapy agent in treating NPC,^{4,5} we hypothesized that the combination of BIBF 1120 with DDP might show greater antitumor activity than when these two inhibitors are administered as single agents. In vivo experiments were performed to verify the earlier hypothesis.

Materials and methods

Cell lines

Three poorly differentiated human NPC cell lines were studied: HNE-1, CNE-2, and HONE-1. HNE-1 was a gift from Professor Kaitai Yao (Southern Medical University, Guangzhou, People's Republic of China). CNE-2 and HONE-1 were gifts from Professor Musheng Zeng (Sun Yat-Sen University Cancer Center, Guangzhou, People's Republic of China). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in a 5% CO₂ humidified atmosphere. Logarithmically growing cells were used in the experiments.

No ethics statement was required from the institutional review board for the use of these cell lines.

Drugs, chemicals, and antibodies

BIBF 1120 was kindly provided by Boehringer Ingelheim (Ingelheim, Germany). DDP (Hansoh Pharmaceuticals, Lianyungang, People's Republic of China) was obtained as a commercial product from our hospital pharmacy. Captisol was obtained from CyDex Pharmaceuticals, Inc. (Lenexa, KS, USA). Fetal bovine serum was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies for VEGF, VEGFR1, and VEGFR2 were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Antibodies against CD31 were obtained from Beijing Zhongshan Jinqiao Biotechnology (Beijing, People's Republic of China).

BIBF 1120 was dissolved in dimethylsulfoxide at a stock concentration of 10 mM, and stored at –20°C, diluting in culture medium just before use. For the in vivo study, BIBF 1120 was dissolved in 0.3% Captisol to a concentration of 10 mg/mL.

Proliferation assay

Tumor cells were cultured in 96-well plates at an appropriate density per well. Varying concentrations of BIBF 1120 were added to the cells 24 hours after plating and incubated for 72 hours, followed by Cell Counting Kit 8 (Dojindo, Tokyo, Japan) assay. The optical density was measured at 450 nm on an enzyme-linked immunosorbent assay reader (SpectraMax M5; Molecular Devices LLC, Sunnyvale, CA, USA). The IC_{50} value was defined as the concentration resulting in 50% lower cell growth compared to that in untreated control cells. The assay was repeated in triplicate in more than three independent experiments.

Tumor xenograft studies

Six- to eight-week-old male BALB/c nude mice, weighing ~16–18 g each, were supplied by Vital River Laboratory Animal Technology Co. Ltd (Beijing, People's Republic of China). All animal experiments were conducted in accordance with “Guidelines for the Welfare of Animals in Experimental Neoplasia” and approved by the Experimental Animal Ethical Committee of Sun Yat-sen University. HNE-1 cells (1×10^7 cells resuspended in 0.2 mL of 0.9% sodium chloride solution, NaCl) were inoculated subcutaneously into the right flank of the nude mice. When tumors reached ~70 mm³ in volume, animals were randomized into four groups ($n=8$ /group): BIBF 1120 group, DDP group, combination group, and control group. BIBF 1120 (10 mL/kg) was administered by oral gavage for 5 days a week. Intraperitoneal injection of DDP (10 mg/kg) was dosed with 0.9% NaCl every week.

Captisol (10 mL/kg), by oral gavage for 5 days per week, and intraperitoneal injection of 0.9% NaCl every week were administered to the controls. The body weight of the mice and tumor sizes were measured and recorded every 3 days. Tumor volume was calculated using the equation $\text{volume (mm}^3\text{)} = \text{length} \times \text{width}^2 \times 0.5$. After 4 weeks of treatment, mice were euthanized, and tumors were harvested, fixed in 10% buffered formalin, and embedded in paraffin.

Immunohistochemical staining of VEGF-VEGFRs

Immunohistochemical (IHC) staining was performed on the formalin-fixed, paraffin-embedded tumor tissue sections. Pathological changes were examined by hematoxylin and eosin staining. The standard avidin–biotin complex peroxidase method was used for IHC detection.²² H-score was used as a measure of IHC. VEGF and VEGFR1/2 were localized in the cytoplasm and cell membrane, respectively. The extension was scored as percentage of positive cells (0%–100%), and the intensity of staining was assessed in comparison to that of a known external positive control (1, weak or below detection; 2, moderate; and 3, strong). H-score was calculated by multiplying the staining intensity and extension at each intensity level.

Microvessel density

Microvessel density (MVD) in the tumor stroma was measured to quantitatively assess angiogenesis. CD31 is usually used as an indicator of angiogenesis, as CD31 is a marker of undifferentiated endothelial cells, which are found in the new blood vessels of malignant tumors. High density of undifferentiated blood vessels indicates a worse prognosis.²¹ MVD was quantified by measuring the number of CD31-positive endothelial cells in the tumors. Five random fields per tumor sample, at 200 magnifications, were captured. MVD was reported as the average number for each group.²³

Statistics

Statistical analysis was performed using SPSS Version 16.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean \pm standard deviation. Comparisons in the *in vivo* studies were analyzed by the unpaired *t*-test with Welch correction. Two-sided $P < 0.05$ was considered statistically significant.

Results

Proliferation-inhibiting effects of BIBF 1120 in NPC cell lines

Three human NPC cell lines, CNE-2, HNE-1, and HONE-1, were treated with BIBF 1120 (0–10 μM) for 72 hours. BIBF

1120 significantly inhibited the growth of these three cell lines in a dose-dependent manner (Figure 1). The IC_{50} values for the effect of BIBF 1120 on HNE-1, CNE-2, and HONE-1 were $4.16 \pm 0.04 \mu\text{M}$, $5.62 \pm 2.64 \mu\text{M}$, and $6.32 \pm 1.18 \mu\text{M}$, respectively. The percentage of inhibition is shown in Table S1.

Antitumor activity of BIBF 1120 *in vivo*

Since the IC_{50} value of BIBF 1120 was found to be the lowest for HNE-1 cells, we further evaluated the efficacy of BIBF 1120 in the HNE-1 xenograft model in nude mice, both as a single agent or in combination with cisplatin. The tumor sizes and weights were evaluated. As shown in Figure 2, BIBF 1120 demonstrated significant growth inhibition of NPC tumors in the HNE-1 xenograft model in nude mice as a single agent; the growth was significantly lower than that in the control group. At the same time, BIBF 1120 showed a greater inhibition of tumor growth in the human NPC cell line xenograft model in nude mice when combined with DDP than in the DDP single-agent group or the control group. Body weight reduction was observed in the DDP single-agent group and combination group, and temporary body weight reduction was observed in the BIBF 1120 single-agent group (Figure 3). No other obvious toxicity was observed in the mice.

BIBF 1120 decreased the expression of VEGF/VEGFRs in the NPC tumor xenograft model

H-scores of VEGF were 107.5 ± 30 , 160 ± 76.2 , 106 ± 43.4 , and 207.5 ± 65 in BIBF 1120, DDP, BIBF 1120 plus DDP, and control groups ($P = 0.059$), respectively (Figure 4A). H-scores of VEGFR1 were 200 ± 37.4 , 165 ± 38.7 , 156 ± 28.8 , and 217.5 ± 45 in BIBF 1120, DDP, BIBF 1120 plus DDP, and

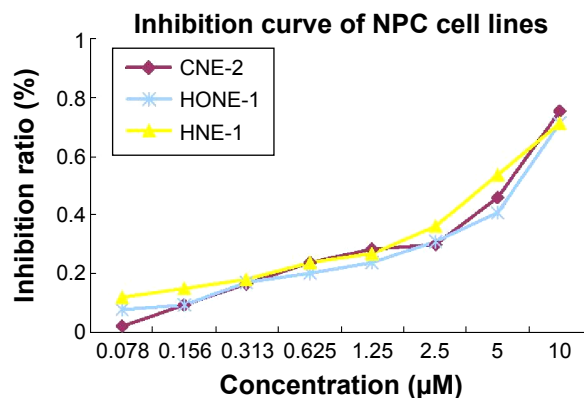


Figure 1 Inhibitory effect of BIBF 1120 on growth of NPC cell lines.

Notes: Cancer cell proliferation was calculated as a percentage of control cell growth. Each point represents the mean of triplicate values \pm SD.

Abbreviations: NPC, nasopharyngeal carcinoma; SD, standard deviation.

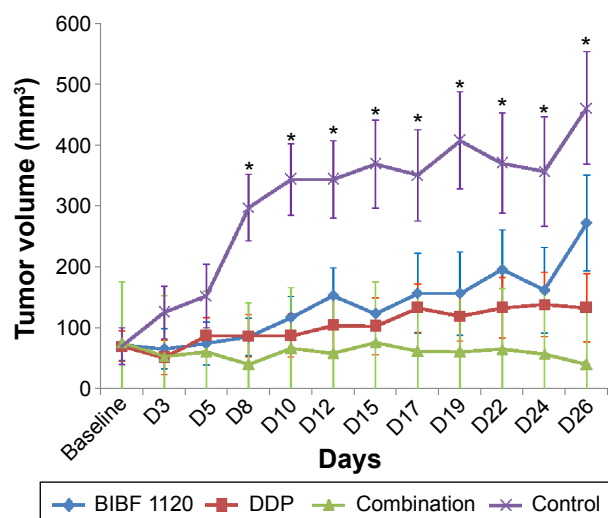


Figure 2 Effect of BIBF 1120, DDP, or combination on the growth of HNE-I xenografts.

Notes: Tumor volume of HNE-I xenografts. Mean tumor volume is shown. Data points represent a mean from six to nine mice; bars, SD. * $P < 0.05$ versus control.

Abbreviations: SD, standard deviation; DDP, cisplatin.

control groups ($P = 0.097$), respectively (Figure 4B). H-score of VEGFR2 was 85 ± 41.2 , 145 ± 50 , 70 ± 52 , and 162.5 ± 26.3 in BIBF 1120, DDP, BIBF 1120 plus DDP, and control groups ($P = 0.023$), respectively (Figure 4C). We found that there was a strong tendency with regard to the expression of VEGF and VEGFR1, despite no statistical significance ($P = 0.059$ for VEGF and 0.097 for VEGFR1). Results showed that BIBF 1120 resulted in significantly lower expression of VEGF/VEGFRs (especially for VEGFR2) in the single-agent

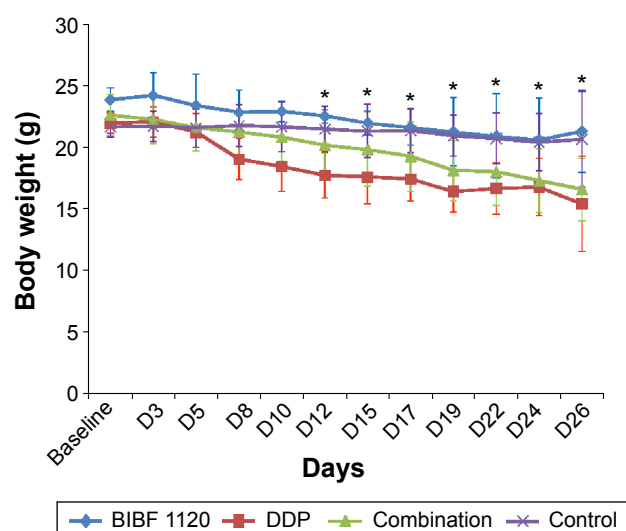


Figure 3 Effect of BIBF 1120, DDP, or combination on the growth of HNE-I xenografts.

Notes: Body weight of HNE-I xenografts. Mean body weight is shown. Data points represent a mean from six to nine mice; bars, SD. * $P < 0.05$ versus control.

Abbreviations: SD, standard deviation; DDP, cisplatin.

group and in the combination group compared to that in the control group.

BIBF 1120 reduced MVD in NPC tumor xenograft model

CD31 staining was used to evaluate MVD. MVDs were 17.9 ± 2.13 , 22.6 ± 6.0 , 16.7 ± 1.6 , and 21.1 ± 3.0 in the BIBF 1120, DDP, BIBF 1120 plus DDP, and control groups ($P = 0.002$), respectively (Figure 5). BIBF 1120 treatment resulted in significantly lower MVD in the HNE-1 xenograft model compared to that in the control group.

Discussion

The efficacy of five antiangiogenic agents including sorafenib, sunitinib, pazopanib, axitinib, and bevacizumab has been investigated in NPC.^{24–29} The administration of sorafenib, sunitinib, and pazopanib as single agents has been shown to be clinically efficacious in treating advanced NPC. The clinical benefit rates ranged from 28% to 54%, most of which reached stable disease, indicating that VEGFR inhibitors might show better efficacy when combined with chemotherapy or radiotherapy.^{25,26,28,30} A Phase II study of bevacizumab combined with chemoradiation revealed good efficacy in treating locally advanced NPC.²⁷ A Phase II study of sorafenib combined with DDP and 5-fluorouracil showed that it was a tolerable and feasible regimen as a first-line therapy in advanced NPC.²⁹ The earlier results indicate that the combination of an antiangiogenic agent with chemotherapy or radiotherapy is mostly safe and more efficacious than treatment with an inhibitor as a single agent.

This is the first preclinical study to investigate the efficacy of BIBF 1120 in NPC. We evaluated the growth inhibitory activity of BIBF 1120 as a single agent and in combination with DDP in human NPC cell lines and in a human NPC xenograft model. Results from the in vitro study showed that the IC_{50} values of BIBF 1120 ranged from $4 \mu M$ to $7 \mu M$ in three poorly differentiated human NPC cell lines. The data indicated that the efficacy of BIBF 1120 in inhibiting proliferation in NPC cell lines was not impressive. Although BIBF 1120 could inhibit NPC cell proliferation at high doses, the average steady-state maximum concentration ($C_{max,ss}$) was 68.6 ng/mL ($0.12 \mu M$, molecular weight 539.62 g/L) in Phase I clinical trials.³⁰ The IC_{50} values of BIBF 1120 for NPC could not be achieved in an in vivo study. As the target of antiangiogenic inhibitors is not the tumor cell itself, we surmised that BIBF 1120 might produce a more powerful effect in xenograft models.

The human NPC xenograft model demonstrated good sensitivity to BIBF 1120 treatment at 100 mg/kg similar

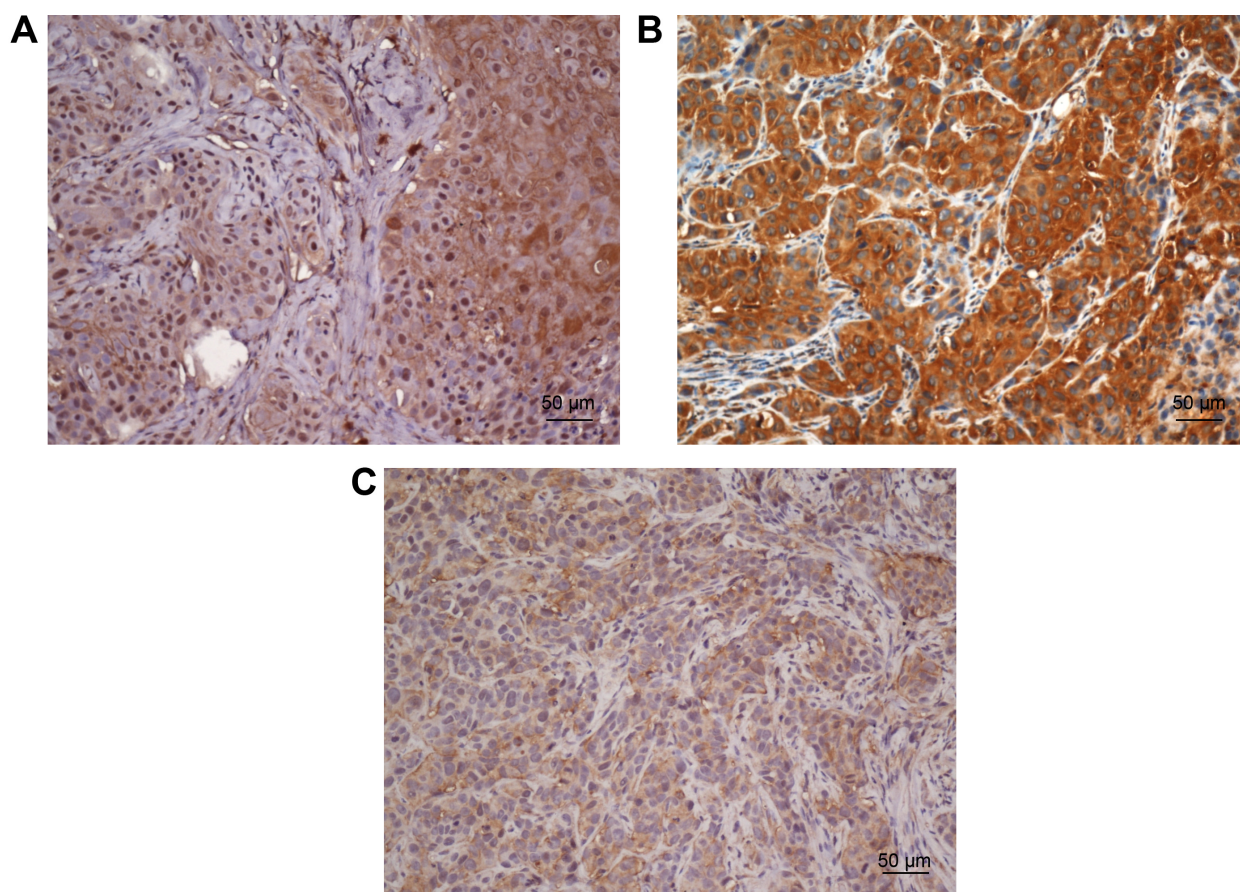


Figure 4 VEGF and VEGFR immunostaining within the tumor of HNE-I NPC xenograft at day 26.

Notes: (A) VEGF, (B) VEGFR1, and (C) VEGFR2. Typical images of strong positive intensities of staining are shown ($\times 200$ magnification).

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; NPC, nasopharyngeal carcinoma.

to the results of previous studies in other tumor xenograft models.^{18–20} BIBF 1120 in combination with DDP resulted in a significant inhibition of tumor growth. However, weight loss was only observed in the combination group and DDP group. We attributed the weight loss to the toxicity of DDP,

indicating a good tolerability of BIBF 1120 at 100 mg/kg repeat dosing in mice. Further studies are warranted to explore the best administration dosage of chemotherapy to fully utilize this potential synergistic effect of BIBF 1120, without increasing toxicity.

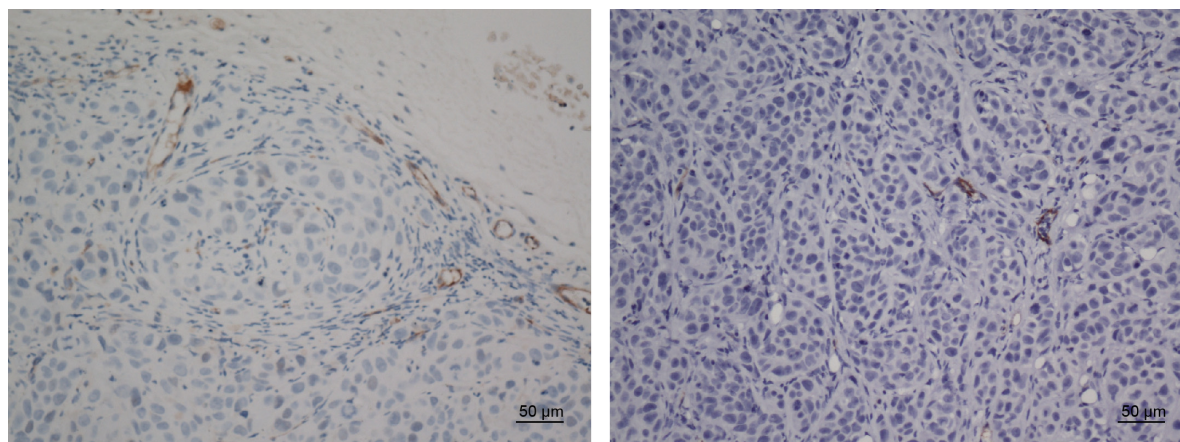


Figure 5 CD31 immunostaining for microvessel density within the tumor of HNE-I NPC xenograft at day 26.

Note: Typical images of control group and BIBF 1120 group are shown ($\times 200$ magnification).

Abbreviation: NPC, nasopharyngeal carcinoma.

Furthermore, we demonstrated that BIBF 1120 resulted in significantly lower expression of VEGF/VEGFRs and MVD compared to that in the control, suggesting that antiangiogenesis represented a pivotal mechanism of action of BIBF 1120 in vivo. The usage of numerous biomarkers of antiangiogenesis remains controversial, including plasma proteins, angiogenesis signaling pathways, MVD, circulating endothelial progenitor/cells, and functional imaging technology.^{31,32} Among these, biomarkers of VEGF/VEGFR pathway have received particular attention, as they enable direct detection of antiangiogenic activity of VEGFR agents. We found that BIBF 1120 mainly inhibits VEGFR2, which was consistent with results from earlier studies.^{18,19} The observed MVD also confirmed the antiangiogenic activity of BIBF 1120, both in the single-agent group and in the combination groups and compared it to that in the monotherapy and control groups.

Conclusion

In conclusion, we showed that BIBF 1120 induces mild inhibition of proliferation in NPC cell lines and a moderate inhibitory effect on tumor growth in an NPC xenograft model. Moreover, data from the NPC xenograft model suggested that concurrent administration of BIBF 1120 with DDP might lead to significantly improved efficacy, but with higher toxicity attributable to DDP. With a modified chemotherapy dosage, we expect that BIBF 1120 plus chemotherapy might be an effective and tolerable regimen for the treatment of NPC.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 The percentage of inhibition of BIBF 1120 on nasopharyngeal carcinoma cell lines

Cell lines	0.078 μM	0.156 μM	0.3125 μM	0.625 μM	1.25 μM	2.5 μM	5 μM	10 μM
CNE-2	0.022	0.092	0.164	0.239	0.285	0.301	0.461	0.751
HONE-1	0.076	0.092	0.171	0.200	0.236	0.311	0.408	0.713
HNE-1	0.118	0.151	0.181	0.236	0.267	0.362	0.536	0.710

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