Evaluation of the Antiangiogenic Effects of 2-Aryl-3-bromoquinolin-4(1*H*)-ones and a NCH₃-4-oxo Derivative

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2-Aryl-3-bromoquinolin-4(1*H*)-ones and 2-aryl-3-bromo-1-methylquinolin-4(1*H*)-one were evaluated for their antiangiogenic effects. Results showed that the 2-aryl-3-bromoquinolin-4(1*H*)-ones (QNHFBr and QNHClBr), and the 3-bromo-2-(4-chlorophenyl)-1-methylquinolin-4(1*H*)-one (QNMeClBr) reduced endothelial cell numbers in an assay of cell proliferation, showing increased cytotoxicity at higher doses. The compounds also inhibited neovessel growth in an *ex vivo* assay of angiogenesis. Furthermore, low levels of proangiogenic factors, namely, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor/placental growth factor (VEGF/PIGF) were obtained in cultures treated with these compounds. Thus, the tested compounds, QNHFBr, QNHClBr, and QNMeClBr exhibit antiangiogenic activity.

Key words quinolone derivative; angiogenesis; basic fibroblast growth factor; vascular endothelial growth factor; placental growth factor

For several years, the 2-arylquinolin-4(1*H*)-ones and their derivatives have been the subject of investigations as a new class of antitumour agents. These compounds are believed to exert their antitumour effect through binding to the colchicine site of tubulin with the resultant inhibition of tubulin polymerization.^{1–3} We are currently interested in the synthesis of 2-aryl-4-quinolone derivatives that are modified by varying the degree of unsaturation and the position of heteroatoms in the heterocyclic ring. We have since prepared a series of 2-aryl-3-halogenoquinolin-4(1*H*)-ones and their NMe-4-oxo derivatives for further studies of chemical transformation and biological activity.

Recently, we reported the synthesis and preliminary antitumour activity of 2-aryl-3-bromoquinolin-4(1*H*)-ones and their 2-aryl-3-bromo-1-methylquinolin-4(1*H*)-one derivatives against the human bladder carcinoma cell line, T24.⁴ This preliminary screening for the medicinal potential of 2-arylquinolin-4(1*H*)-ones provided a basis for further evaluation of antiangiogenic activities of some of the compounds that showed antitumour activity. Tumours require an efficient blood supply to survive and grow beyond a critical size.⁵ The formation of new blood vessels from existing microvessels, termed angiogenesis, has been cited as a dominant feature in tumor growth and metastasis.^{5–7} The process of angiogenesis is characterized by alterations in a number of endothelial cell functions, including endothelial cell proliferation and capillary morphogenesis, each of which can serve as a potential target for antiangiogenic strategies.^{6,8,9} The process of angiogenesis is also mediated by a number of growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and placental growth factor (PIGF).⁷ These growth factors have been reported to promote endothelial cell proliferation and angiogenesis *in vitro*.^{7,8}

Although the antitumour activities of quinolone derivatives are well documented,^{1–3} to our knowledge their effects on angiogenesis have not been thoroughly studied. This prompted us to probe selected 2-aryl-3-bromoquinolin-4(1*H*)-ones and their NMe-4-oxo derivatives for potential

antiproliferative activity on endothelial cells. The effects of these compounds on neovessel growth *in vitro* were investigated. The levels of bFGF and the VEGF/PIGF heterodimer in endothelial cell culture supernatants were also measured.

MATERIALS AND METHODS

Cell Culture Experiments were conducted on human dermal microvascular endothelial cells (HMEC-1), a generous gift from the Centre for Disease Control, U.S.A. The cell line was maintained as monolayer cultures in a 37 °C incubator in a humidified atmosphere containing 5% CO₂. HMEC-1 were cultured in MCDB-131 medium (Lab Specialist Services, Cape Town, South Africa), supplemented with 1% penicillin–streptomycin (Highveld Biologicals, Sandringham, South Africa), 10% fetal bovine serum (Highveld Biologicals), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, U.S.A.), 1 µg/ml hydrocortisone (Sigma-Aldrich), and 10 ng/ml epidermal growth factor (Sigma-Aldrich). Estrogen receptor positive breast cancer cells (MCF-7), human osteoblastic cells (MG63), and the human bladder carcinoma cells (T24) were maintained in Dulbecco's minimum essential medium supplemented with 1% penicillin–streptomycin (Highveld Biologicals) and 10% fetal bovine serum (Highveld Biologicals).

Preparation of Quinolone Derivatives 3-Bromo-2-(4-fluorophenyl)quinolin-4(1*H*)-one (**1**), 3-bromo-2-(4-chlorophenyl)quinolin-4(1*H*)-one (**2**), and 3-bromo-2-(4-chlorophenyl)-1-methylquinolin-4(1*H*)-one (**3**) used in this investigation were synthesized as described in our previous communication.⁴ The test compounds were prepared in dimethylsulfoxide (DMSO) at concentrations of 0–10 µM.

Proliferation Assay Cell proliferation studies were undertaken using HMEC-1, MCF-7, MG63, and T24 cells. Cells were seeded in 24-well culture plates at a density of 10000 cells per well and exposed to varying concentrations of test compounds. Control cells were exposed to DMSO. 2-Methoxyestradiol (2-ME), an antiangiogenic microtubule-disrupting drug,^{10,11} was used in this investigation as a posi-

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tive control. Growth was terminated after 48 h by fixation with 1% glutaraldehyde in PBS for 15 min. Cells were then stained with a 1% solution of crystal violet (in H₂O) for 30 min. The chromophore was extracted with 500 μ l of 0.1% Triton X-100 per well.¹²⁾ The absorbance of the samples was read at 570 nm on an ELx 800 Universal Microplate Reader.

Measurement of bFGF Endothelial cells HMEC-1 were cultured in 24-well plates as described above and exposed to DMSO or varying doses of QNHFB (1), QNHCB (2), QNMCB (3) or 2-ME for 24 h. The levels of bFGF in cell culture supernatants were measured using a Quantikine human bFGF ELISA kit (R & D, Minneapolis, U.S.A.), according to the manufacturer's instructions. Briefly, 100 μ l of standard, control or sample was added to each well in a 96-well plate and incubated for 2 h. Each well was aspirated and washed four times with wash buffer. After aspirating the wells, 200 μ l of bFGF conjugate was added to each well and the plates incubated for another 2 h. The wells were aspirated, and 200 μ l of substrate solution was added to each well. Subsequently, 500 μ l of stop solution was added to each well. The optical density of each well was read at 450 nm on an ELx 800 Universal Microplate Reader.

Measurement of VEGF HMEC-1 were cultured in 24-well plates for 24 h and then exposed to DMSO or varying doses of QNHFB (1), QNHCB (2), QNMCB (3) or 2-ME. After 24 h of exposure to test compounds, the levels of VEGF/PIGF were measured in HMEC-1 supernatants using a human VEGF/PIGF heterodimer immunoassay kit (R & D, Minneapolis, U.S.A.), according to the manufacturer's instructions. Briefly, 50 μ l of standard, control or sample was added to each well in a 96-well plate and incubated for 2 h. Each well was aspirated and washed four times with wash buffer. After aspirating the wells, 200 μ l of VEGF/PIGF conjugate was added to each well and the plates were covered with an adhesive strip and incubated for another 2 h. The wells were aspirated, and 200 μ l of substrate solution was added to each well. Subsequently, 50 μ l of stop solution was added to each well. The optical density of each well was read at 450 nm on an ELx 800 Universal Microplate Reader.

In Vitro Angiogenesis Two Sprague-Dawley male rats (6–8 weeks old) were terminated by suffocation using isoflurane. The aortas of the rats were cut and transferred to a petri-dish with medium. Rat aorta rings were prepared and cultured as previously described.⁷⁾ Briefly, the aorta from each rat was cleaned, cut into 1 mm wide rings, and then transferred into a petri dish with fresh cell culture medium. Rings were embedded in fibrin gel in 24-well culture plates and maintained in MCDB-131 medium supplemented with 0.3 mg/ml amino caproic acid and 1% penicillin–streptomycin. Cultures were then exposed to DMSO (control), quinolone derivatives or 2-ME for 10 d. Medium and compounds were renewed every 2 d. Cultures were monitored for growth every second day. Images were captured using a Nikon digital camera. Areas of neovessel growth were calculated using Photoshop software. Prior approval by the University of Pretoria's Animal Use and Care Committee (project V025-08) was obtained for this study.

Statistical Analysis Data were analysed using one-way analysis of variance (ANOVA), followed by a *post hoc* test (Bonferroni's method). Results are presented as means $\pm$ standard deviation (S.D.). Significance was designated at

$p < 0.05$.

RESULTS

The structures of quinolone derivatives tested in this study, namely, QNHFB (1), QNHCB (2), and QNMCB (3) are represented in Fig. 1. These test compounds inhibited HMEC-1 proliferation (Fig. 2), with IC₅₀ values of approximately 0.027 μ M for QNHFB, 0.100 μ M for QNHCB, and 0.316 μ M for QNMCB (Table 1). 2-Methoxyestradiol (2ME), an antiangiogenic agent that is currently in phase II clinical trials, was employed in this study as a positive control. 2-ME inhibited HMEC-1 proliferation with an IC₅₀ value of about 0.25 μ M. The IC₅₀ value for each compound was calculated as the concentration of test compound resulting in a 50% reduction of viable cells compared to control cells. The IC₅₀ values for the compounds obtained after treating various cell lines are shown in Table 1. Angiogenesis was assessed using an *ex vivo* rat aorta ring assay. Neovessels were observed in control (Fig. 3A) and treated cultures (Figs. 3B–E) compared to control cultures. However, the area of

Fig. 1. Chemical Structures of Test Compounds

(A) 2-Arylquinolin-4(1H)-ones 1 (R=F) and 2 (R=Cl); (B) 2-(4-chlorophenyl)-1-methylquinolin-4(1H)-one 3.

Table 1. The Effects of Test Compounds on the Proliferation of Various Cell Lines Represented as IC₅₀ Values

Cell line	IC ₅₀ value (μ M)		
	QNHFB (1)	QNHCB (2)	QNMCB (3)
HMEC-1	0.027 $\pm$ 1.3	0.100 $\pm$ 1.0	0.316 $\pm$ 1.8
MCF-7	4.205 $\pm$ 1.6	3.857 $\pm$ 1.4	3.985 $\pm$ 2.5
T24	0.240 $\pm$ 1.1	0.221 $\pm$ 0.8	0.328 $\pm$ 0.3
MG63	0.195 $\pm$ 4.9	0.250 $\pm$ 2.3	0.320 $\pm$ 2.9

Fig. 2. Inhibitory Effects of Quinolone Derivatives on Cell Proliferation

Antiproliferative effects of test compounds QNHFB (1), QNHCB (2), QNMCB (3) and 2-methoxyestradiol (positive control) on HMEC-1 were assessed using a colorimetric crystal violet staining assay. Results represent the number of viable cells as a percentage of control cells (% HMEC-1) calculated from the mean of three wells $\pm$ S.D. Data shown are representative of three independent experiments.

Fig. 3. Inhibition of *in Vitro* Angiogenesis by Quinolone-Derivatives in an Aorta Ring Assay

(A—E) Photographs showing the inhibitory effects of test compounds on neovessel growth. (A) Untreated (control) culture; (B) aorta ring treated with 10 μM 2-ME (positive control); (C) aorta ring treated with 10 μM QNHFB (1); (D) aorta ring treated with 10 μM QNHClBr (2); and (E) aorta ring treated with 10 μM QNMeClBr (3). Bar 250 μm. (F) Quantitative analysis of *in vitro* angiogenesis was undertaken by calculating the means of the vascularised areas. Each data point represents the mean ± S.E. (standard error) of three wells. Data shown are representative of two separate experiments.

Fig. 4. Inhibitory Effects of QNHFB (1), QNHClBr (2) and QNMeClBr (3) on HMEC-1 bFGF and VEGF/PlGF Heterodimer Secretion

Levels of (A) bFGF and (B) VEGF/PlGF heterodimer in HMEC-1 supernatants 24 h after exposure of cells to various concentrations of test compounds and to 2-ME were measured using ELISA kits as described in Materials and Methods. Results shown represent the mean ± S.D. from three independent experiments conducted in triplicate for each treatment condition. * $p < 0.05$ vs. control.

neovessel growth was decreased considerably in treated cultures (Figs. 3B—E). Quantitative analysis revealed that test compounds inhibited new vessel growth in a dose-dependent manner, and with similar potency as 2-ME (Fig. 3F).

To determine the effects of the test compounds on bFGF secretion, the levels of this proangiogenic growth factor were measured. At concentrations below 0.01 μM of test compounds 1, 2 and 3, there were marginal differences in the levels of bFGF secreted by treated and untreated HMEC-1 (Fig. 4A). However, at higher doses (0.1 μM for compounds 1 and 2), and 1 μM for compound 3, the compounds inhibited bFGF secretion, with compound 1 exhibiting more potency. Low levels of the VEGF/PlGF heterodimer were secreted in cultures treated with test compounds compared to control (DMSO-treated) cultures (Fig. 4B). On the other hand, 2-ME showed greater potency in inhibiting the secretion of VEGF/PlGF than test compounds 1—3 (Fig. 4B).

DISCUSSION

Pathological angiogenesis is a key event associated with continued growth of tumours, and contributes to the hematogenous spread of cancer cells.^{6,7)} This dependence of tumour growth on angiogenesis has led to the development of novel therapies that are targeted at the tumour vasculature.^{13–16)} The antitumour effects of quinolones and their derivatives have been reported in a number of studies.^{1,2)} However, studies on the effects of these compounds on an-

giogenesis have not received much attention. As an initial study into the antiangiogenic effects of these novel compounds, the effects of 2-aryl-3-bromoquinolin-4(1*H*)-ones (compounds **1** and **2**) and 2-aryl-3-bromo-1-methylquinolin-4(1*H*)-one (compound **3**) were evaluated for possible antiangiogenic activity. Studies were performed to assess the effects of test compounds on endothelial cell proliferation, an important endothelial cell function associated with angiogenesis. Test compounds inhibited HMEC-1 proliferation with varying potency and in a dose-dependent manner, with IC₅₀ values of 0.027–0.316 μ M. The most potent of these compounds, QNHFBBr (**1**), inhibited cell proliferation at a concentration of 0.01 μ M, and exhibited an increased antiproliferative effect at higher doses.

The ability of a compound to inhibit endothelial cell proliferation does not provide sufficient proof of antiangiogenic activity. Consequently, we investigated the effects of test compounds on the proliferation of various cancer cell lines. The observed results revealed that compounds (**1**) and (**2**) were more potent in inhibiting HMEC-1 than MCF-7, T24 or MG63 cells. However, marginal differences were observed in the antiproliferative effects of compound **3** on the various cell lines, indicating that the antiproliferative effects of this compound might not be selective to endothelial cells. Based on the above observations, we extended our studies and evaluated the effects of quinolone derivatives using the aorta ring assay, a three-dimensional culture model of angiogenesis. The results revealed that test compounds inhibited new vessel formation, with compound (**1**) exhibiting more potency than the other two derivatives.

Two studies have shown that bFGF and the VEGF/PlGF heterodimer promote angiogenesis *in vitro* and *in vivo*.^{17,18} Both bFGF and VEGF have been implicated in the pathogenesis of vascular tumours of infancy.¹⁹ On the other hand, taxol, a chemotherapeutic drug and a potent inhibitor of angiogenesis, has been shown to induce a decline in bFGF secretion by human small cell lung carcinoma cells (SW2).²⁰ These literature observations prompted us to further evaluate the effects of test compounds on bFGF and VEGF/PlGF secretion. The observed results showed that the test compounds induced a decrease in the secretion of bFGF and VEGF/PlGF by cultured HMEC-1.

The results of this investigation therefore showed that test compounds (**1**), (**2**) and (**3**) exhibit antiangiogenic activity. These compounds presumably exert this activity by inhibiting one of the cell functions that are important in angiogenesis, namely, endothelial cell proliferation, and by decreasing bFGF and VEGF/PlGF secretion by endothelial cells. At equimolar concentrations, compound (**1**) and 2-ME inhibited bFGF secretion with comparable potency. The fluoro derivative (**1**) also inhibited VEGF/PlGF secretion, albeit with less potency than 2-ME. The most pronounced antiangiogenic activity in all the tests was observed for the 4-fluorinated derivative (**1**) and activity decreased when fluorine was replaced with a chlorine atom. On the other hand, significantly less activity was observed for the *N*-methylated derivative (**3**). The presence of a fluorine atom in a molecule is known to have a profound effect on the biological, chemical and physical

properties of such derivatives. Fluoroquinolones, for example, are known to show unique pharmacological properties²¹ and the analogous 2-arylquinolin-4(1*H*)-ones bearing fluorine atoms at the 2' or 3' position of the phenyl ring were previously found to exhibit increased antitumour activity.^{2,3} On the other hand, decreased activity in the case of the NMe derivative **3** can be attributed to the importance of the amine hydrogen (NH) in quinolone analogues with biological activity.^{22,23} The 4'-fluorophenyl NH-4-oxo derivative (**1**) could thus serve as an excellent lead compound to provide good models for further design of potent fluorinated 2-arylquinolone derivatives with potential to serve as angiogenesis inhibitors.

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