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Vertical VEGF targeting: A combination of ligand blockade with receptor tyrosine kinase inhibition

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ABSTRACT

The aim of this study was to examine the anti-tumour effects of dual vertical VEGF targeting consisting in the association between bevacizumab, a VEGF-depleting drug, and the VEGF receptor antityrosine kinase AZD2171.

Mice bearing human head and neck CAL33 xenografted tumours were treated once daily for 11 d with either vehicle (controls), AZD2171 (2.5 mg/kg/day, p.o.), bevacizumab (5 mg/kg/day, i.p.) or the bevacizumab-AZD2171 combination.

The AZD2171-bevacizumab combination produced additive effects on tumour growth and reduced the number of proliferating cells relative to control. Bevacizumab did not influence tumour vascular necrosis whilst AZD2171 ($p = 0.01$) and the combination ($p = 0.01$) increased it. The number of mature tumour cells decreased significantly with the combination treatment only ($p = 0.001$), which induced the largest increase in the Bax/Bcl2 ratio (up to 25-fold) and a progressive 3-fold decrease in HIF1-α expression between 24 h and 192 h. The present data indicate that there is no redundancy in targeting the same angiogenic pathway with the presently tested clinically applicable drugs. The study provides a strong rationale for future clinical trials.

1. Introduction

The initiation, the growth and the development of new blood vessels are the critical features of tumour growth.[1] An increasing number of drugs that inhibit VEGF signalling are being used in the treatment of cancer, commonly in combination with chemotherapy.[2] However, treatments using single angiostatic agents have yet to demonstrate complete inhibition of neovascular growth in the clinic, and thus far have only delayed tumour growth.[3] In addition, although these agents are generally well tolerated, they may be accompanied by distinct adverse effects including hypertension and proteinuria.[4] Multiple anti-angiogenic targeting strategies may lead to reduce doses of single agents, alleviate toxic effects and optimise anti-tumour efficacy. Combinations of anti-angiogenic agents can be considered from two angles. On the one hand, there is 'horizontal inhibition', which targets complementary pro-angiogenic pathways such as those involving VEGF receptors and PDGF receptors. On the other hand, there is the 'vertical inhibition' strategy, which consists in an optimal blockade of a given critical pathway. VEGF axis is a representative application for the latter strategy.[5]

Many of these approaches are ongoing and require careful early clinical trials for toxicity assessment and also dedicated

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pre-clinical studies. The purpose of this study was to examine the anti-tumour effects of dual vertical VEGF targeting consisting in the association between bevacizumab, a representative VEGF-depleting drug, and the antityrosine kinase AZD2171. Bevacizumab is the first VEGF inhibitor approved by the FDA for systemic use in cancer. This monoclonal antibody is currently approved in conjunction with chemotherapy in advanced colorectal cancer[6,7] and unresectable non-small cell lung cancer.[8] AZD2171 is a highly potent (IC50 < 1 nM) ATP-competitive inhibitor of recombinant VEGF receptor-2 (VEGFR-2) tyrosine kinase *in vitro*.[9] This compound also shows potent activity against VEGFR-1 (IC50 = 5 nM) and VEGFR-3 (IC50 $\leqslant$ 3 nM).

Tumour vasculature is amongst the key targets for innovative management of head and neck cancer.[10] We recently reported on the anti-tumour efficacy of AZD2171 alone or in combination with gefitinib and irradiation in human head and neck tumour xenograft CAL33 which secretes VEGF.[11] This study examines the respective impacts of each drug alone or in combination with CAL33 tumour growth considering the effects not only on tumour vasculature but also on the tumour itself for proliferation and apoptosis.

The possibility of early and transient tumour vasculature normalisation under the effects of VEGFR-2 and VEGF blockade has been previously described.[12] This alternative mode of action has recently been confirmed for bevacizumab[13] and AZD2171.[14] In order to examine more closely the early changes in tumour vasculature during treatment, most study parameters were thus examined daily over a 10-d period after commencement of treatment.

2. Material and methods

2.1. Chemicals

AZD2171. was kindly provided by AstraZeneca and bevacizumab (Avastin® from Roche) by our institution's pharmacy. Working solutions were prepared as follows: AZD2171 (375 mg l^{-1}) was suspended in 0.9% NaCl, 0.01% Tween 80 and bevacizumab (750 mg l^{-1}) diluted in 0.9% NaCl. For both drugs the concentrations were adjusted so as to include the daily dose in 0.2 ml of drug suspension. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and glutamine were purchased from Whittaker (Verviers, Belgium). Foetal bovine serum (FBS) was obtained from Dutscher (Brumath, France).

2.2. Tumour cell line

CAL33, a cell line of human head and neck origin (HNSCC from the base of the tongue) was obtained from our institution (Centre Antoine Lacassagne). This cell line exhibits high EGFR levels (33,800 fmol mg^{-1} protein high affinity sites determined by Scatchard assay, Ref.[15] and produces VEGF (measured by ELISA using the DVE00 kit from Quantikine, R§D; Cercina Onesto, CNRS-UMR6543 Nice, personal communication).

Cells were maintained as a monolayer culture in DMEM supplemented with 10% FBS v/v, 2 mM glutamic acid, 50,000 units l^{-1} penicillin and 80 μM streptomycin in a humidified incubator (Sanyo, Japan) at 37 °C in an atmosphere containing 8% CO_2. Batches of 15×10^6 cells were frozen in FBS supplemented with 5% dimethyl sulfoxide (DMSO) v/v in advance for injection into mice. Shortly before injection, cells were thawed and suspended in Ringer lactate.

2.3. Mice

Six-week-old female NMRI nude mice were purchased from Janvier laboratories (Le Genet sur Isle, France) and received subcutaneous inoculation in the right flank of 0.75×10^6 cells suspended in 200 μl of Ringer lactate ($n = 35$ per treatment condition). There were five animals per cage with food and water *ad libitum*. Following tumour cells injection (day 0), animal weight and tumour volume were monitored on day 10, on day 13 (beginning of treatment), and on days 14, 15, 16, 20 and 23 (end of treatment). Additional monitoring was performed until animal sacrifice. Tumours were collected 24 h, 48 h, 72 h, 96 h, 144 h and 196 h after the beginning of treatment (5 animals sacrificed at each time). As no signs of suffering appeared during the experiment, no animal had to be sacrificed before the end of the study. For ethical reasons, animals were sacrificed when tumours reached 3000 mm^3. Time of final sacrifice was day 35 for the control group, day 39 for the AZD2171 group and day 52 for the bevacizumab and combination groups (five animals sacrificed at each time). Animals were sacrificed by cervical disruption. Animal experiments were performed in accordance with the regulations of the United Kingdom Co-ordinating Committee on Cancer Research guidelines.[16]

2.4. Treatment

Doses of AZD2171 and bevacizumab were chosen in accordance with preliminary experiments so that each drug given alone exerted only partial effects on tumour growth. Mice bearing CAL33 tumours were treated once daily for 11 d, from days 13 to 23 (the mean tumour volume on day 13 was 250 mm^3). Treatment consisted of either vehicle (controls), AZD2171 (2.5 mg/kg/d, p.o.), bevacizumab (5 mg/kg/d, i.p.) or the bevacizumab-AZD2171 combination (concomitant administration at the same doses as for each drug alone). Effects of treatment on tumour growth were evaluated as previously described by us: fractional tumour volume (FTV) for each treatment group was calculated as the ratio between the mean tumour volumes of treated and untreated tumours.[17] This was done for treatment a (FTVa), for treatment b (FTVb) and for treatment a + b (FTV a + b). The expected FTV for the a+b combination was defined as observed FTVa × observed FTVb. We then computed a combination ratio (CR) defined as the expected FTVa + b/observed FTVa + b. Supra-additive effects corresponded to CR > 1.2 and infra-additive effects to CR < 0.8. Strictly additive effects were considered in the case of 0.8 < CR < 1.2.

2.5. Tumour measurements

In all experiments, there were 5 animals examined per time point and group of treatment.

– *Growth analysis*: Tumour length and width were measured using a calliper rule. Tumour volume was calculated as $\Pi/6 \times \text{length} \times \text{width}^2$. Tumours were removed surgically at

Table 1 – Tumour necrosis score

0	1	2	3
Absence of necrosis	Few pre-necrotic features microvacuolisation, enhancement of eosinophilia, kystic degeneration or ballooning	Numerous pre-necrotic features, small necrosis areas	Necrosis area accounting for more than 30% of the tumour

the time of sacrifice. Half of the tumour was directly frozen in liquid nitrogen for protein analysis and the other half fixed in paraformaldehyde overnight.

– *Histology*: After fixation, tumours were embedded in paraffin. Morphologic studies were processed on 3 μm paraffin sections (one sagittal section through the centre of the tumour) stained with standard haematoxylin phloxine safran (HPS). Differentiation, necrosis, vascular density and vascular necrosis were evaluated on HPS slides. Tumour cell maturation was evaluated by the percentage of well-differentiated and/or ortho-keratinised cells (clearer pink-coloured cytoplasm with a clearly delineated cobblestone-like cytoplasm frame and visible intercellular junctions) as opposed to undifferentiated (darker amphiphilic cytoplasm with a greater nucleocytoplasmic ratio and smaller, darker, more rounded cells) tumour cells. The number of mature tumour cells was evaluated using the product of the tumour volume by the percentage of mature cells. Tumour necrosis was evaluated as indicated in Table 1.

– Tumour marker.

2.5.1. *Immunohistochemistry (IHC)*

Ki67, HIF1 α vascular density and vascular necrosis (vasculature was characterised by VEGFR2 labelling on endothelial cells) IHC analyses were performed by a pathologist who visually excluded stroma, using the following primary antibodies: MiB1 (Dako Corporation, Trappes, France), [HI alpha67-sup] ab463 (Abcam, Cambridge, United Kingdom) and 55B11 (Cell Signalling Technology – Ozyme, Saint Quentin Fallavier, France) for Ki67, HIF1 α and VEGFR2, respectively. One sagittal section through the centre of the tumour was analysed.

Ki67 and VEGFR2 immunostaining was performed with a BenchmarkXT immunostainer (Ventana Medical Systems, Illkirch, France). Sections were scored semi-quantitatively by two pathologists using light microscopy.

For Ki67 and HIF1 α the percentage of tumour cells with a nuclear staining was evaluated. The number of proliferating or HIF1 α expressing cells was assessed using the product of the tumour volume by the percentage of proliferating or HIF1 α expressing cells, respectively.

The scores for vascular density and vascular necrosis are defined in Table 2.

2.5.2. *Western blot analyses (Bax/Bcl2)*

Frozen tumours were pulverised in a liquid nitrogen-cooled Thermovac grinder. The resulting powders were homogenised in 10 volumes of a 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM sodium molybdate, phosphatase inhibitor cocktail 2 with a 1/100 dilution and protease inhibition cocktail 2 with a 1/100 dilution, both from Sigma (Saint Quentin Fallavier, France). The homogenates were centrifuged for 1 h at 105,000 g (+4 °C), and the supernatants (cytosols) were used for protein determination by immunoblotting. Total protein content was measured using the bicinchoninic acid assay. The apoptosis-related markers Bax/Bcl2 ratio was determined by Western blot and normalised by Raf as a loading control using the following primary antibodies Anti Bax from Upstate – Millipore (Molsheim, france), Anti Bcl2 and Anti Raf from Pharmingen (San Diego, California). Western blots were quantified using the Chemi Doc imager from BioRad.

2.5.3. *VEGF secretion analysis*

Human VEGF-A secreted by CAL33 xenografted tumours was determined in tumour cytosol by ELISA using the DVE00 kit (Quantikine, R§D).

All the above-mentioned parameters were analysed at 24 h, 48 h, 72 h, 96 h, 144 h, and 196 h after the beginning of treatments.

2.6. *Statistics*

Analysis of tumour volume (log 10-transformed data) was performed according to a General Linear Model (GLM) that included the treatment effect (control *versus* bevacizumab *versus* AZD2171 *versus* combination), the time effect and the treatment x time interaction. Therefore, comparisons between treatment groups were performed by post-hoc tests. For other quantitative variables, i.e. the number of mature cells, proliferating cells at the tumour border, hypoxic cells at the tumour border, VEGF secretion and Bax/Bcl2 expression, the comparison between treatment groups was ana-

Table 2 – Grading score for vascular density and vascular necrosis

	0	1	2	3
Vascular density	No vessel	Few vessels	Many vessels	–
Vascular necrosis	Intact vessels clearly delineated	Alteration of vascular walls	Vessel necrosis, extravasation of red cells	More than 75% of vessel necrosis and no intact vessel

lysed by means of the non-parametric Mann-Whitney test, by considering all studied times. For ordinal values such as vascular density, tumour necrosis and vascular necrosis, the χ^2-test was performed. The two-side significance level was 0.05 for GLM analysis. For Mann–Whitney and χ^2-tests, multiple comparisons ($k = 5$) were performed. Thus, the two-side significance level (α) was fixed at 0.0102 according to the formula $(1-\alpha)^k = 0.95$, so that the global type I error remained 0.05. Statistics were drawn up on SPSS software, version 15.0.

3. Results

3.1. *Impact on tumour growth*

During the treatment period, AZD2171, bevacizumab and their combination significantly reduced tumour growth relative to control (Fig. 1A). The combination was more potent than bevacizumab alone ($p = 0.001$), and its efficiency was close to that of AZD2171 ($p = 0.049$). After the end of treatment, tumours of the AZD2171 group promptly escaped whereas the AZD2171-bevacizumab group exhibited a marked and longer-lasting effect, close to that of the bevacizumab alone (Fig. 1B). At the end of treatment (day 23), tumour volumes for AZD2171 and bevacizumab groups were 48% and 33% that of control, respectively. At distance from treatment, tumour volumes relative to controls at day 32 were 49% with AZD1271 and 25% with bevacizumab (Fig. 1B). The AZD2171-bevacizumab combination produced additive effects (the mean of 5 CR computed between days 12 and 32 was 0.8 ± 0.09). Globally the differential effect between bevacizumab and the combination was evident during the treatment phase and lost at distance (from day 32 to the end of the observation phase).

3.2. *Treatment toxicity*

Global toxicity was evaluated by monitoring animal body weight. As indicated by the steadily increased body weight, all treatments were well tolerated by the animals (unshown data).

3.3. *Treatment effects at cellular and molecular levels*

All effects were measured during the treatment period (24–192 h).

3.3.1. *Tumour cell proliferation*

The number of proliferating cells was assessed by Ki67 labelling at the border of the tumour-growing masses. All positive Ki67 cells belonged to the undifferentiated cell sub-population. As compared to controls, all treatments significantly reduced tumour cell proliferation ($p < 0.002$, Fig. 2). The number of proliferating cells was not statistically different between single drug treatments and their combination. However, the combination rapidly (as early as 24 h) and clearly (by approximately 3-fold) reduced the number of proliferating cells relative to control, and this effect remained constant until 192 h after the beginning of treatment (Fig. 2).

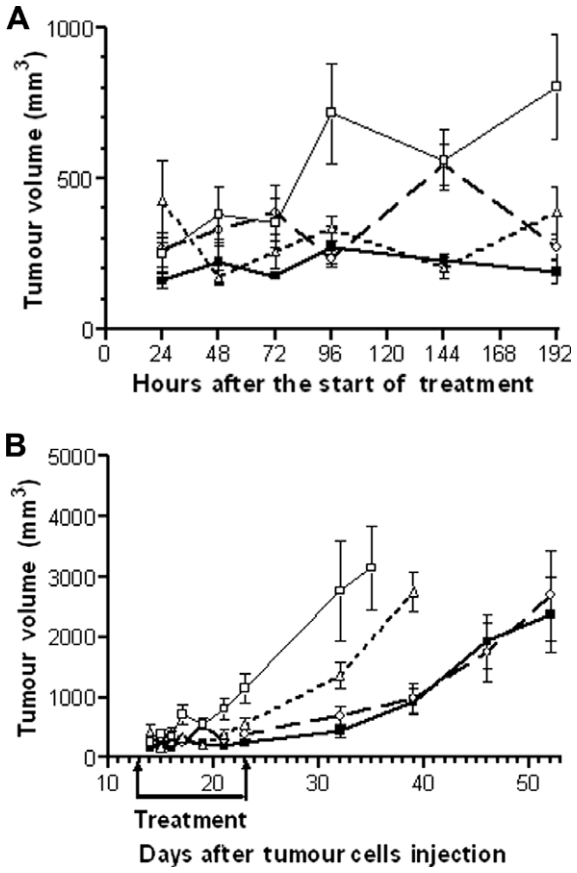

Fig. 1 – Effect of AZD2171 (Δ - - - -Δ), bevacizumab (○ —— ——○), their combination (■ ——— ■) and vehicle (□ ——— □) on tumour growth (mean tumour volume ± SD of 5 tumours per treatment group). (A) During the treatment period (treatment administered from T_0 up to 264 h). AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p = 0.025$; combination *versus* control: $p < 0.001$; bevacizumab *versus* combination: $p = 0.001$; AZD2171 *versus* combination: $p = 0.049$ (GLM analysis). (B) Over the entire follow-up period, i.e. between day 14 after tumour cell injection (24 h after the start of treatment) and day 52 (last follow-up time corresponding to animal sacrifice when tumour volumes reached 3000 mm^3 in the more efficient treatment groups). AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p = 0.003$; combination *versus* control: $p < 0.001$; bevacizumab *versus* combination: $p = 0.035$; AZD2171 *versus* combination: $p = 0.001$ (GLM analysis).

3.3.2. *Tumour cell maturation*

During the growth of control tumours, the number of mature tumour cells increased 3-fold between 72 h and 192 h (Fig. 3). The number of mature cells remained stable with the combination treatment only ($p = 0.001$ as compared to control). At 144 h, a transient increase in the number of mature cells was observed with bevacizumab (Fig. 3).

3.3.3. *Tumour apoptosis*

Apoptosis was assessed by determining the Bax/Bcl2 ratio. All treatments significantly increased tumour apoptosis as compared to controls ($p < 0.001$, Fig. 4). Single drugs produced a

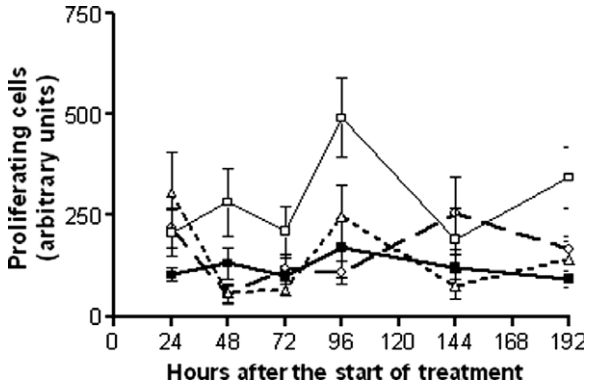

Fig. 2 – Effect of AZD2171 (Δ - - - -Δ), bevacizumab (o —— —— o), their combination (■ ——— ■) and vehicle (□ ——————□) on the number of positive Ki67 staining cells at the periphery of the tumours during the treatment period (mean number of proliferating cells ± SD of 5 tumours per treatment group). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p = 0.001$; bevacizumab *versus* control: $p = 0.002$; combination *versus* control: $p < 0.001$; bevacizumab *versus* combination: $p = 0.48$; AZD2171 *versus* combination: $p = 0.69$ (Mann–Whitney tests).

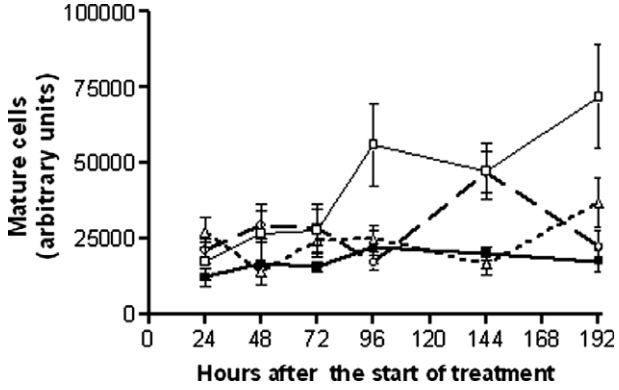

Fig. 3 – Effect of AZD2171 (Δ - - - -Δ), bevacizumab (o —— —— o), their combination (■ ——— ■) and vehicle (□ ——————□) on the number of mature cells during the treatment period (mean number of well-differentiated and/or ortho-keratinised cells ± SD of 5 tumours per treatment group). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p = 0.023$*; bevacizumab *versus* control: $p = 0.093$; combination *versus* control: $p = 0.001$; bevacizumab *versus* combination: $p = 0.002$; AZD2171 *versus* combination: $p = 0.029$* (Mann–Whitney tests). *not significant at the α risk fixed at 0.01.

significant but small increase in the Bax/Bcl2 ratio (<7-fold that of control), whereas the drug combination produced a larger increase in the Bax/Bcl2 ratio, up to 25-fold that of control at 144 h after the beginning of treatment; the latter effect disappeared rapidly after 48 h.

3.3.4. *Tumour necrosis*

Tumour necrosis was the lowest in the control group. This observation in control cells may reflect the spontaneous necrosis accompanying tumour growth. As soon as 24 h following the beginning of treatment, necrosis was greater in treated animals than in controls, and the necrosis score increased over time in all treated groups (Fig. 5). As compared to untreated tumours, tumour necrosis was significantly greater in the AZD2171 ($p < 0.001$) and combination ($p = 0.001$) groups only. For bevacizumab, increased tumour necrosis was observed at 144 h after starting treatment (Fig. 5). In treated groups, the necrosis increase was not corroborated by an increase in tumour volume, and probably reflected treatment efficiency.

3.3.5. *Tumour vasculature*

As compared to controls, vascular density was significantly reduced by AZD2171 ($p < 0.001$) and the AZD2171-bevacizumab combination ($p < 0.001$) (Fig. 6). The addition of bevacizumab did not improve the effects observed with AZD2171 alone. A complementary pattern was observed in vascular necrosis: AZD2171 ($p = 0.010$) and the combination ($p = 0.011$) increased vascular necrosis relative to controls (Fig. 7). Here again, bevacizumab did not influence vascular necrosis. The effect of AZD2171 peaked 96 h after the beginning of treatment, returning subsequently to a pattern comparable to controls.

3.3.6. *VEGF secretion by the tumour*

Both drugs administered alone significantly induced VEGF secretion ($p < 0.005$, Fig. 8). The highest VEGF secretion was observed in the AZD2171 group, whatever the observation time, with a maximum occurring at 144 h after starting AZD2171 (13-fold relative to controls). In contrast, the drug combination eliminated this phenomenon (Fig. 8).

3.3.7. *Tumour cell hypoxia (HIF1α)*

In the control group, the number of HIF1 α -stained cells doubled between 24 h and 192 h (Fig. 9). All treatments significantly decreased the number of hypoxic cells ($p < 0.004$). The most noticeable effects were observed with the drug combination which induced a progressive 3-fold decrease between 24 h and 192 h (Fig. 9). Interestingly, the drug combination produced a similar impact on tumour proliferation and on tumour hypoxia.

4. Discussion

VEGF is the most potent promoter of tumour-associated angiogenesis, and represents one of the most attractive and relevant targets for therapeutic applications. Combination studies are currently evaluating in renal cell carcinoma the feasibility of associating bevacizumab with inhibitors such as sorafenib[5] of the tyrosine kinase domain of the VEGF receptor. The aim of a 'vertical blockade' is to obtain a more complete pattern of VEGF inhibition. To this end, pre-clinical studies are needed in order to examine closely the respective effects of these drugs administered alone or in combination. A recent experimental study by Dorell and colleagues[18] elegantly demonstrated synergistic effects with another strategy of 'horizontal inhibition' combining angiostatic molecules that in this case target distinct facets of the angiogenic process. The results showed complete

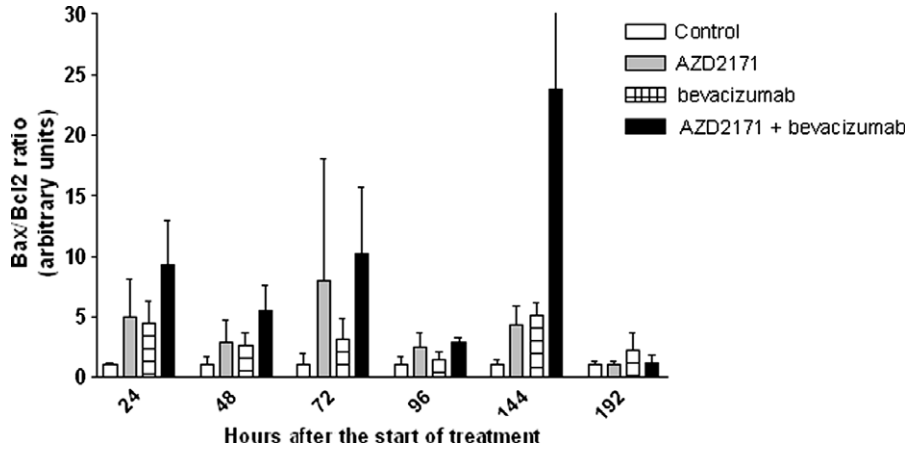

Fig. 4 – Effect of AZD2171 (▭) bevacizumab (▤), their combination (▬) and vehicle (▭) on the Bax/Bcl2 expression ratio during the treatment period (mean ratio ± SD of 5 tumours per treatment group). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p < 0.001$; combination *versus* control: $p < 0.001$; bevacizumab *versus* combination: $p = 0.16$; AZD2171 *versus* combination: $p = 0.072$ (Mann–Whitney tests).

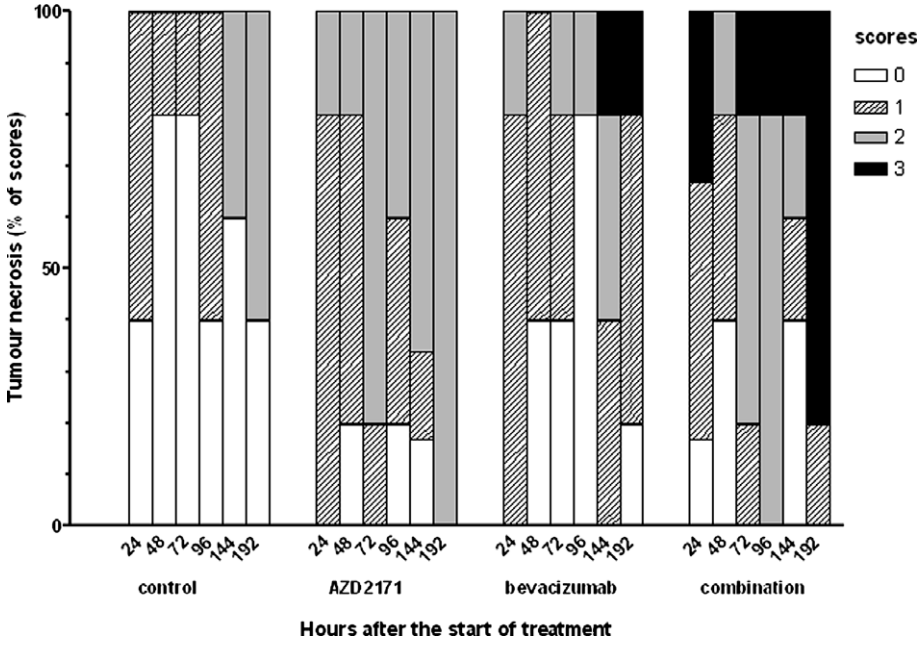

Fig. 5 – Effect of vehicle, AZD2171, bevacizumab and their combination on tumour necrosis during the treatment period (see Table 1 in the Material and Methods section). Results are expressed as the percentage of the tumour necrosis score for each studied time (5 tumours per treatment group). Scores are represented as follows: score 0 (▭), score 1 (▩), score 2 (▤) and score 3 (▬). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p < 0.052$; combination *versus* control: $p = 0.001$; bevacizumab *versus* combination: $p = 0.077$; AZD2171 *versus* combination: $p = 0.005$ (χ^2-tests).

inhibition of vascular growth on the basis of ischaemic retinopathy and tumour vasculature.[18] It was the purpose of this study to consider 'vertical inhibition' of the VEGF pathway and, to this end, to examine a combination of bevacizumab, a reference VEGF inhibitor with clinically proven activity,[6–8] with AZD2171, which is a highly potent ATP-competitive inhibitor of VEGFR-2.[9] Several findings emerge from this work regarding the impact of treatments alone or in combination on tumour growth, on tumour angiogenesis and histology and on changes in tumour markers of proliferation and apoptosis.

Regarding tumour growth itself, it is noteworthy that during the phase of treatment the combination bevacizumab-AZD2171 led to additive effects (Fig. 1). Shortly after the end of the treatment period, there was a phase of tumour regrowth which was stronger in mice treated by the VEGFR-2 inhibitor in comparison with bevacizumab and the drug combination (Fig. 1). This observation is in line with the evolution

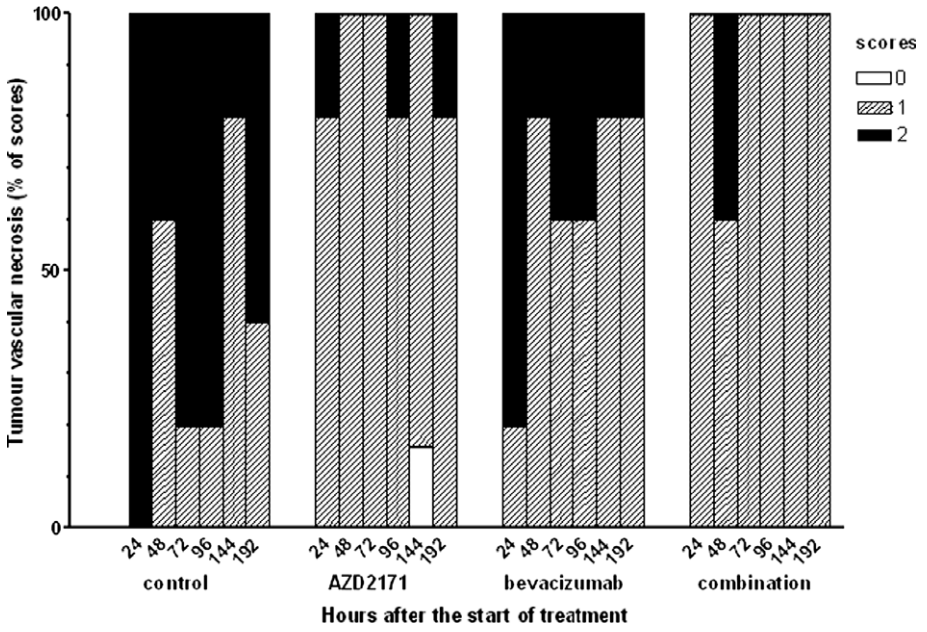

Fig. 6 – Effect of vehicle, AZD2171, bevacizumab and their combination on tumour vascular density during the treatment period (see Table 2 in the Material and Methods section). Results are expressed as the percentage of the grading score for each studied time (5 tumours per treatment group). Scores are represented as follows: score 0 (▭), score 1 (▩), score 2 (■). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p < 0.070$; combination *versus* control: $p < 0.001$; bevacizumab *versus* combination: $p = 0.005$; AZD2171 *versus* combination: $p = 0.66$ (χ^2-tests).

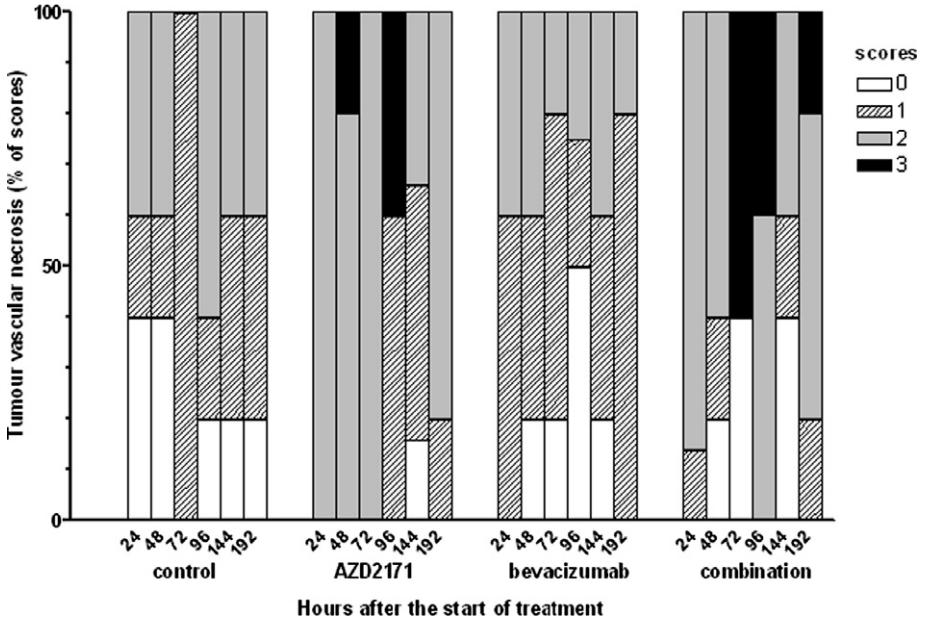

Fig. 7 – Effect of vehicle, AZD2171, bevacizumab and their combination on tumour vascular necrosis during the treatment period (see Table 2 in the Material and Methods section). Results are expressed as the percentage of the grading score for each studied time (5 tumours per treatment group). Scores are represented as follows: score 0 (▭), score 1 (▩), score 2 (▭) and score 3 (■). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p = 0.010$; bevacizumab *versus* control: $p < 0.65$; combination *versus* control: $p = 0.011$; bevacizumab *versus* combination: $p = 0.002$; AZD2171 *versus* combination: $p = 0.20$ (χ^2-tests).

of both Ki67 labelling and the apoptosis-related ratio Bax/Bcl2 which were the lowest and the highest, respectively, consecutive to the association of the two anti-angiogenic drugs (Figs. 2 and 4). Moreover, this pattern was confirmed by the results concerning tumour necrosis which increased steadily during the treatment phase (Fig. 5). Regarding the results of anti-

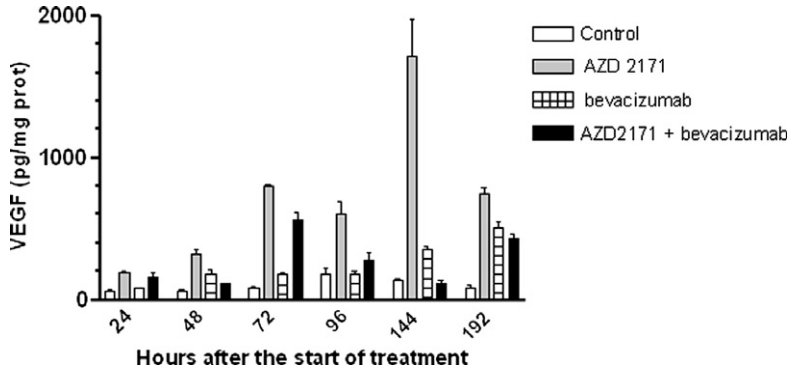

Fig. 8 – Effect of AZD2171 (▭), bevacizumab (▤), their combination (▬) and vehicle (▭) on VEGF secretion by the CAL33 tumour xenograft (mean ratio ± SD of 5 tumours per treatment group). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p < 0.005$; combination *versus* control: $p < 0.003$; bevacizumab *versus* combination: $p = 0.93$; AZD2171 *versus* combination: $p < 0.001$ (Mann–Whitney tests).

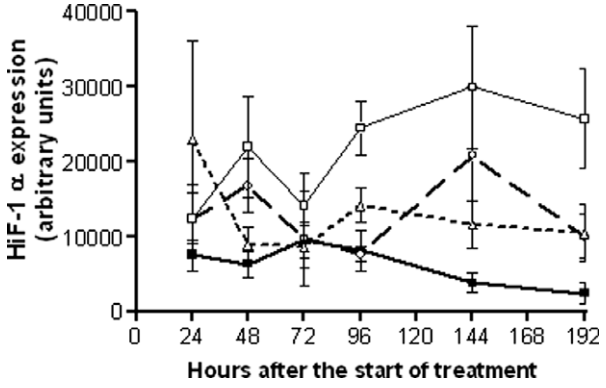

Fig. 9 – Effect of AZD2171 (Δ - - - -Δ), bevacizumab (○ —— —— ○), their combination (■ ▬▬▬ ■) and vehicle (□ ▭▭▭▭ □) on the number of HIF1 α -expressing cells during the treatment period (mean number of HIF1 α -expressing cells ± SD of 5 tumours per treatment group). Treatment was administered from T_0 up to 264 h. AZD2171 *versus* control: $p < 0.001$; bevacizumab *versus* control: $p = 0.004$; combination *versus* control: $p < 0.001$; bevacizumab *versus* combination: $p = 0.025$*; AZD2171 *versus* combination: $p = 0.03$ (Mann–Whitney tests). * not significant at the α risk fixed at 0.01.

angiogenic treatments on tumour vasculature itself (VEGFR-2 staining), the two drugs behaved differently, the changes induced by AZD2171 being much more marked and occurring earlier during the treatment phase than those caused by bevacizumab (Fig. 6). We thus noted a distinction between the marked anti-angiogenic activity of each drug maintained over time after treatment arrest and the capacity for tumour re-growth following the cessation of treatment.

The compensatory up-regulation of pro-angiogenic factors following angiostatic therapy was examined through the changes in VEGF tumour expression (Fig. 8). Interestingly, here again, the two anti-angiogenic agents differed in their action with the AZD2171 treatment regimen inducing a marked but transient rebound in tumour VEGF expression. This could explain the fact that treatment by AZD2171 was followed by the sharpest tumour re-growth following the therapeutic phase (Fig. 1). It is also conceivable that this tumour re-growth phenomenon is not only related to tumour-induced neo-angiogenesis but can also be initiated by the overproduction of the growth factor VEGF with autocrine phenomenon considering that VEGF receptors are carried not only by endothelial cells but also by tumour cells.[19] Recent experimental and clinical data have reported on the presence of VEGF increases in tumour and/or peripheral circulation under anti-angiogenic treatment.[20–22] Interestingly, the drug combination totally abolished this VEGF tumour expression, and there followed a moderate re-initiation of tumour growth as compared to the AZD2171.

One of the most striking and unexpected findings of this study was the different impacts of VEGF-targeted treatments on tumour histology. Clearly, more necrosis was present following VEGFR-2 targeting by AZD2171 as compared to bevacizumab (Fig. 5). One explanation for these observations may lie in the fact that AZD2171 is also able to directly target cancer cells[23], and this capability may thus help generate greater tumour necrosis, in conjunction with the expected anti-angiogenic effect of this drug. The presence of vascular necrosis may help account for the overproduction of VEGF which could be related to tumour hypo-vascularisation inducing HIF-1 alpha.[24,25] With the drug combination, a parallel was observed between the evolution of these 3 parameters (tumour vascular necrosis, VEGF expression, HIF-1 α expression) with a peak at 72 h during treatment (Figs. 7–9). The supra-additivity between anti-angiogenic therapy and chemotherapy is still not completely understood, and one of the plausible causes of this phenomenon may be tumour vasculature normalisation. Recent studies have shown that both AZD2171 and bevacizumab induce this vasculature normalisation effect.[13,14] We thus planned to examine the so-called 'therapeutic window' created by the normalisation of tumour vasculature. This accounts for the repeated daily tumour investigations during the treatment phase of this study. Although not particularly marked, there were several time-related impacts of each drug during the treatment period. Normalisation of tumour vasculature was hard to assess during the treatment period, and there were no evident time-related and reversible changes in vasculature density during treatment, irrespective of the drug applied (Fig. 6). For each drug considered, however, there were concom-

itant and transient changes in the parameters analysed in this study. For instance, under bevacizumab, at 144 h following the start of treatment, there were treatment peaks in tumour regrowth, in tumour cell proliferation, in the proportion of tumour mature cells (Fig. 3) and in the expression of HIF-1α. It is possible that this short time window could be favourable for a combination with a chemotherapeutic agent. In contrast, in the presence of the drug AZD2171 at 96 h following the beginning of treatment, we noted a maximum and reversible impact on tumour vasculature necrosis accompanied by maximum tumour production of VEGF (compensatory phenomenon) which was observed two days later (144 h following treatment start).

In summary, the present data indicate that there is no redundancy in targeting the same angiogenic pathway, VEGF, with complementary therapeutic tools such as a monoclonal antibody targeting VEGF itself and a strong VEGFR-2 tyrosine kinase inhibitor. Additive anti-tumour effects were observed on the tumour xenograft model used in the study. Interestingly, although blocking the same angiogenic factor, each drug produced different effects on the tumour. These preclinical data are reinforced by the absence of any noticeable side-effect in the treated animals, and thus provide a strong rationale following the encouraging early clinical trials recently reported with this treatment strategy.[26]

Conflict of interest statement

None declared.

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REFERENCES

1. Zhong H, Bowen JP. Antiangiogenesis drug design: multiple pathways targeting tumor vasculature. *Curr Med Chem* 2006;**13**:849–62.
2. Petersen I. Antiangiogenesis, anti-VEGF(R) and outlook. *Recent Results Cancer Res* 2007;**176**:189–99.
3. Verhoef C, de Wilt JH, Verheul HM. Angiogenesis inhibitors: perspectives for medical, surgical and radiation oncology. *Curr Pharm Des* 2006;**12**:2623–30.
4. Kamba T, McDonald DM. Mechanisms of adverse effects of anti-VEGF therapy for cancer. *Br J Cancer* 2007;**96**:1788–95.
5. Rini BI, Rathmell WK. Biological aspects and binding strategies of vascular endothelial growth factor in renal cell carcinoma. *Clin Cancer Res* 2007;**15**:741s–6s.
6. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;**350**:2335–42.
7. Giantonio BJ, Catalano PJ, Meropol NJ, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol* 2007;**25**:1539–44.
8. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;**355**:2542–50.
9. Wedge SR, Kendrew J, Hennequin LF, et al. AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. *Cancer Res* 2005;**65**:4389–400.
10. Le QT, Giaccia AJ. Therapeutic exploitation of the physiological and molecular genetic alterations in head and neck cancer. *Clin Cancer Res* 2003;**9**:4287–95.
11. Bozec A, Formento P, Lassalle S, et al. Dual inhibition of EGFR and VEGFR pathways in combination with irradiation: antitumour supra-additive effects on human head and neck cancer xenografts. *Br J Cancer* 2007;**97**:65–72.
12. Jain RK. Normalizing tumor vasculature with antiangiogenic therapy: a new paradigm for combination therapy. *Nat Med* 2001;**7**:987–9.
13. Dickson PV, Hamner JB, Sims TL, et al. Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenogerafts results in improved delivery and efficacy of systemically administered chemotherapy. *Clin Cancer Res* 2007;**13**:3942–50.
14. Batchelor TT, Sorensen AG, di Tomaso E, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell* 2007;**11**:83–95.
15. Dassonville O, Formento JL, Francoual M, et al. Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. *J Clin Oncol* 1993;**11**:1873–8.
16. Workman P, Twentyman P, Balkwill F, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). *Br J Cancer* 1998;**77**:1–10.
17. Guerin O, Formento P, Lo Nigro C, et al. Supra-additive antitumor effect of sunitinib malate (SU11248, Sutent® combined with docetaxel. A new therapeutic perspective in hormone refractory prostate cancer. *J Cancer Res Clin Oncol* 2008;**134**:51–7.
18. Dorrell MI, Aguilar E, Scheppke L, et al. Combination angiostatic therapy completely inhibits ocular and tumor angiogenesis. *Proc Natl Acad Sci U S A* 2007;**104**:967–72.
19. Zhong XS, Liu LZ, Skinner HD, et al. Mechanism of vascular endothelial growth factor expression mediated by cisplatin in human ovarian cancer cells. *Biochem Biophys Res Commun* 2007;**358**:92–8.
20. Schmitz V, Vilanueva H, Raskopf E, et al. Increased VEGF levels induced by anti-VEGF treatment are independent of tumor burden in colorectal carcinomas in mice. *Gene Ther* 2006;**13**:1198–205.
21. Norden-Zfoni A, Desai J, Manola J, et al. Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. *Clin Cancer Res* 2007;**13**:2643–50.
22. Faivre S, Delbaldo C, Vera K, et al. Safety, pharmacokinetic, and antitumor activity of SU11248, a novel oral multitarget tyrosine kinase inhibitor, in patients with cancer. *J Clin Oncol* 2006;**24**:25–35.
23. Takeda M, Arao T, Yokote H, et al. AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. *Clin Cancer Res* 2007;**13**:3051–7.
24. Kim SJ, Rabbani ZN, Dewhirst MW, et al. Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. *Lung Cancer* 2005;**49**:325–35.
25. Koukourakis MI, Giatromanolaki A, Polychronidis A, et al. Endogenous markers of hypoxia/anaerobic metabolism and anemia in primary colorectal cancer. *Cancer Sci*. 2006;**97**:582–8.
26. Feldman DR, Kondagunta GV, Ronnen EA, et al. Phase I trial of bevacizumab plus sunitinib in patients (pts) with metastatic renal cell carcinoma (mRCC). Proceedings of ASCO 2007, abstract 5099.