



Bevacizumab and Sorafenib Modulate P-Glycoprotein Function *In Vitro* and Bevacizumab Increases *In Vivo* Sorafenib Plasma Concentrations in Mice

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Abstract

Overexpression of P-glycoprotein (P-gp) is associated with multidrug resistance. Since sorafenib (NEXAVAR®) is a P-gp (an efflux protein of ATP-binding cassette family) substrate, we tested whether bevacizumab (AVASTIN®), a monoclonal antibody directed toward VEGF (Vascular Endothelial Growth Factor) and sorafenib could modulate P-gp functionality.

In vitro two human ovarian carcinoma cells (IGROV1) overexpressing or weakly expressing P-gp were used. Bevacizumab and sorafenib effects on P-gp functionality were evaluated by measuring doxorubicin intracellular accumulation.

In vivo study was to document whether bevacizumab could modify sorafenib disposition in mice. Therefore, concentrations of sorafenib were determined by HPLC in plasma of mice bearing a human colorectal carcinoma xenograft when sorafenib is given orally (5 mg/kg) on day 4, alone or after a pretreatment with bevacizumab (5 mg/kg IP) on days 1 and 3.

In vitro a significant doxorubicin accumulation and reversion of doxorubicin resistance in P-gp expressing cell lines were observed with bevacizumab or sorafenib pretreatment

In vivo, sorafenib AUC was 1.44 fold significantly higher in bevacizumab pretreated group and C_{max} was 1.35 fold higher in bevacizumab-pretreated group. Mean residence time of sorafenib increased in the presence of bevacizumab, this increase reflects an improvement of sorafenib bioavailability after bevacizumab pretreatment.

We may conclude that bevacizumab pretreatment decreases P-gp functionality and increases doxorubicin intracellular accumulation *in vitro* and sorafenib plasma concentrations *in vivo*.

Keywords: Sorafenib, bevacizumab, P-glycoprotein, pharmacokinetics, ABCB1

1. Introduction

Sorafenib (NEXAVAR®) is an oral multitarget tyrosine kinase inhibitor (CRAF, BRAF, V600E BRAF, FLT3, and C-KIT); it also inhibits tumor vasculature (CRAF, VEGF-R1, R2 and R3, PDGFR b). It is indicated in the treatment of advanced HCC, advanced renal cell carcinoma after failure of previous treatment with interferon alfa or interleukin-2 in patients in whom these therapies were inadequate [1,2,3]. It is indicated also in the treatment of

progressive, locally advanced or metastatic differentiated thyroid carcinoma.

However sorafenib is a drug with a high inter and intra-individual pharmacokinetic variability [4,5,6]. It is the substrate of efflux transporters, breast cancer resistance Protein (BCRP) and P-glycoprotein (ABCB1) [7], with a potential risk of under exposure of patients treated with this drug. Sorafenib-resistant cells showed increased metastatic and invasive ability, with a higher expression of P-glycoprotein (P-gp), compared to that of the parental cells [8].

M. R. Bani et al [9] showed that tumor endothelial cells (TEC) may acquire drug resistance, a characteristic that is maintained *in vitro*. There is evidence that TEC are more resistant to chemotherapeutic drugs, substrates of ATP-binding cassette (ABC) transporters. TEC express P-glycoprotein (encoded by ABCB1), while no difference with other ABC transporters was revealed compared to normal endothelia. In intratumor vessels, they might contribute to create a "bloodtumor barrier", interfering with the delivery of chemotherapy to neoplastic cells. TEC show reduced sensitivity to some chemotherapeutic drugs compared to normal endothelial cells. Increasing evidence suggests that TEC are the first line of tumor chemoresistance.

Inhibition of VEGF biological activity by bevacizumab, a humanized monoclonal antibody (immunoglobulin G 1) could improve the activity of a co-administrated chemotherapy [10].

The use of bevacizumab to patients with metastatic colorectal cancer (mCRC) provides progression-free survival (PFS) and overall survival (OS) advantages when combined with cytotoxic chemotherapy in the first and second line settings [11].

In recurrent glioblastoma patient the combination of bevacizumab with sorafenib has resulted in high objective response rate [12].

Since sorafenib is a multikinase inhibitor targeting several serine/threonine and tyrosine kinases and including all VEGF-receptors, Raf, PDGF receptor B, and FGF receptor 1, the J.M. Hubbard *et al* have shown that dual antiangiogenic treatment combined with cytotoxic therapy may provide prolonged disease stabilization for select patients with advanced GI malignancies [13].

Philippe V. et al have studied the clinical influence of sorafenib and bevacizumab on pancreatic cancer volume and have shown that pancreatic volume decreases statistically significantly under treatment with both the multi-tyrosine kinase inhibitor sorafenib and the angiogenesis inhibitor bevacizumab [14].

Several other clinical trials have focused on the combination of sorafenib and bevacizumab in the treatment of patients with metastatic colorectal cancer [15].

Since sorafenib is a multikinase inhibitor targeting several serine/threonine and tyrosine kinases and including all VEGF-receptors, Raf, PDGF receptor B, and FGF receptor 1, the combination of bevacizumab and sorafenib might provide a more complete blockade of proangiogenic pathways as well as inhibit mechanisms of bevacizumab resistance like overexpression of P-gp in endothelial cells

In the present work we studied potential interactions between anti-angiogenic drugs (sorafenib, and bevacizumab) and P-gp functionality. We studied the effect of sorafenib and /or bevacizumab on doxorubicin resistant cells (IGROV1-DXR) over expressing P-gp.

In vivo we studied how pretreatment with bevacizumab of human colorectal carcinoma bearing mice modifies plasma sorafenib concentrations.

2. Material and Methods

2.1. Drugs

Doxorubicin was purchased from Teva (Paris France), verapamil from Abbott (Paris France) bevacizumab (AVASTIN®) [25mg/mL] were purchased from Pfizer (Montrouge, France) and Roche Registration Limited (Welwyn Garden City, United Kingdom) laboratories, respectively.

2.2. Chemicals

Sorafenib (Nexavar®) was purchased from Bayer Pharma® (Berlin, Germany), erlotinib (internal standard, IS) was purchased from LC® laboratories (Woburn, USA). Dullbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), phosphate-buffered saline (PBS), penicillin, streptomycin, zeocin, hygromycin B. were purchased from Gibco Invitrogen (Cergy-Pontoise France). Acetonitrile and methanol (liquid-chromatography grade), tetrabutyl ammonium and trifluoroacetic acid were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), Acetic Acid were purchased from VWR BDH Prolabo (Haasrode, Belgium), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Sigma-Aldrich Chemical Co, Saint-Quentin, France) ethyl acetate, acetonitrile and dimethylsulfoxide (DMSO) from VWR® (Fontenay-sous-Bois, France). Ammonium acetate was purchased from Sigma® (St. Louis, MO, USA). Deionized purified water was prepared in our laboratory using DirectQ UV® System (Millipore SAS®, Molsheim, France).

2.3. Cell lines

The IGROV1 cells were obtained from Dr. Bernard (Bernard et al., 1985) at the CNRS 8126 laboratory in Villejuif, France.

2.3.1. Establishment of sensitive and resistant doxorubicin cell lines:

IGROV1-p and IGROV1-DXR

A doxorubicin-resistant cell line (IGROV1-DXR), which overexpressed P-gp, was derived from the parental drug-sensitive cell line (IGROV1-p). IGROV1-p cell line was derived from a stage III human ovarian carcinoma. The characterization of the parental sensitive IGROV1-p has been reported previously [16,17]. The IGROV1-DXR cells were selected from IGROV1-p cells by continuous exposure of these cells to increasing concentrations of doxorubicin in a gradual step-wise manner, up to 0.1 µg / ml for doxorubicin. Cell lines were grown as adherent monolayers supplemented with 10% (v/v) FCS, penicillin (100 U/ml) and streptomycin (100 µg / ml) at 37°C in a humidified atmosphere of 5% CO₂ in air. IGROV1-DXR cells were maintained in medium supplemented with doxorubicin (0.1 µg/ml).

2.3.2. Doxorubicin accumulation assay measured by flow cytometric analysis FACS

P-gp function assessment.

Cell-based doxorubicin transport assays were performed in IGROV1-p and IGROV1-DXR cell lines. Cells were incubated in DMEM with doxorubicin alone (10 µM) plus 5% FBS during 2 h at 37 °C in order to compare doxorubicin accumulation in the two cell lines. After incubation with doxorubicin, cells were washed twice with PBS and dissociated with 0.05% Trypsin-EDTA buffer. Cells were re-suspended in DMEM plus 10% FCS then centrifuged. After washing three times with PBS to eliminate extracellular doxorubicin, fluorescence due to doxorubicin intracellular accumulation was measured by flow cytometer analysis (acquisition of data for 10,000 cells in channel 2)

2.3.3. Effect of bevacizumab on P-gp function in IGROV1-DXR lines.

Effect of bevacizumab was tested at 2 different concentrations (1 mg/ml and 5mg/ml) co-incubated in DMEM with doxorubicin 10 µM and 5% FCS during 2 h at 37°C. Bevacizumab effect was also tested on IGROV1-p cells. Verapamil was used during doxorubicin incubation as a positive control. P-gp function assessed by doxorubicin accumulation was expressed as percentage of doxorubicin accumulation through the following equation:

100 x (MFI bevacizumab – MFI control)/MFI control.

2.3.4. Effect of sorafenib on P-gp function in IGROV1-DXR lines.

Effect of sorafenib was tested at 2 different concentrations (5 mg/ml and 10 mg/ml) co-incubated in DMEM with doxorubicin 10 µM and 5% FCS during 2 h at 37°C. Sorafenib effect was also tested on IGROV1-p cells. Verapamil was used during doxorubicin incubation as a positive control. P-gp function assessed by doxorubicin accumulation was expressed as percentage of doxorubicin accumulation through the following equation:

100 x (MFI sorafenib – MFI control)/MFI control.

2.4. Human colorectal carcinoma bearing mice

In this study, was used a xenograft model originating from a human tumour collection, established under the CReMEC projet: the CR-IGRO16P primary xenograft. The patient, from whom the tumour originated, was a woman, with an adenocarcinoma of the sigmoid colon with ovarian metastases. The sample was directly derived from the primary tumour.

The CR-IGRO16P tissue was cut into small pieces. These tissues were subcutaneously implanted into Female Foxn1nu CD-1 nude mice flanks. These mice were purchased from Animal and Veterinary Resources, Institut Gustave Roussy, IFR54 (Villejuif, France). Mice were housed under standard laboratory sterile conditions, with sterile water and regular sterile (gamma-irradiated) chow provided ad libitum in a 12-h/12-h light/dark cycle at a 21-23°C temperature. Anesthesia was induced with 5% isoflurane and maintained with 2.5% isoflurane in air. When the tumour had reached 2-3 cm in diameter, it was sampled and cut into small pieces to obtain the second-passage model, which was used in the study.

2.5. Sorafenib pharmacokinetic studies

Six weeks old male nude mice (20–30 g) were purchased from Animal and Veterinary Resources of Gustave Roussy Institut (IGR Villejuif, France). Mice were randomized into two groups of 21 mice each. The first group received orally sorafenib at the dose of 5 mg/kg. The second group was pre-treated intraperitoneally with bevacizumab at the dose of 5 mg/kg on days 1 and 3 and one hour later received orally sorafenib at 5 mg/kg.

Three mice randomly chosen were anaesthetized with isoflurane and sacrificed by cervical dislocation, their blood was collected by cardiac puncture in heparinized tubes at 0.5, 1, 2, 3, 6 and 16 h after sorafenib administration. After centrifugation at 3,000 rpm for 5 minutes at 4°C, plasma was transferred to polypropylene tubes and stored at -20°C until assays.

The animals were treated in accordance with the European Committee standards concerning the care and use of laboratory animals. The experimental protocol was approved by Local Animal Experimentation Committee (N°26, Ministère de la Recherche et de l'Enseignement Supérieur).

2.6. Sorafenib quantification in plasma

Sorafenib concentrations were measured by a HPLC method coupled with UV-visible detector validated according to the recommendations for bioanalytical method validation [21].

We used Shimadzu® chromatography system with UV detector and Labsolutions® chromatography workstation (Shimadzu Corporation®, Kyoto, Japan). Chromatographic separation was achieved on a C18 ultrasphere ODS® column (250 mm x 4.6 mm, 5 µm; Phenomenex®, Pecq, France) associated with a guard column packed with the same phase.

The calibration was linear in the range of 50-700 ng/mL. The accuracy, within-assay and between-assay precision of the method was 96.9-104.0%, 3.4-6.2% and 7.6-9.9%, respectively. Three sorafenib quality controls were prepared in drug-free plasma: low (50 ng/mL), medium (300 ng/mL) and high (500 ng/mL). The composition of the mobile phase was 40% ammonium acetate (20 mM) and 60% acetonitrile, delivered at a flow rate of 1.2 mL/min.

2.7. Data analysis

Data were analysed separately for each mouse group, average concentration at each sampling time was used. A non-compartmental analysis was performed using WINNONLINE professional version 5.2 software (Pharsight, Mountain View, CA, USA). The mean maximum concentrations (C_{max}) and the times necessary to reach it (T_{max}) were evaluated from experimental curves. Terminal half-lives (t_{1/2}) were calculated from the respective terminal rate constants (K_e), estimated as the slope of the log-linear terminal portion of the mean concentration vs time curve, by linear regression analysis. The mean areas under the concentration–time curves (AUC) were calculated by the trapezoidal method from 0 to the last concentration–time point. AUC of the two groups were compared using Bailer's method [18].

Statistical significance was set at $p < 0.05$. Results are expressed as mean ± standard error to mean (SEM). Under the null hypothesis which indicates that mean AUC are equal, this statistics follows a normal distribution. The null hypothesis is rejected if $|Z_{obs}|$ is greater than 1.96.

3. Results

3.1. In vitro study

3.1.1. Effect of sorafenib on doxorubicin intracellular accumulation

Decrease of P-gp activity by sorafenib was measured by the amount of doxorubicin intracellular accumulation in IGROV1-p and IGROV1-DXR when cultured in presence of sorafenib. Significant dose-dependent increase of doxorubicin uptake in IGROV1-DXR cells was observed when sorafenib was added to the culture at concentrations of 5.0 µM (* $P < 0.05$; n=3) and 10.0 µM (** $P < 0.01$; n=3) as compared to IGROV1-DXR cells not treated with sorafenib (control cells). In IGROV1-p cells, a non statistically significant increase of doxorubicin uptake by cells was observed with sorafenib at the concentration of 10.0 mg/ml as compared to control cells. Treatment by the P-gp inhibitor, verapamil, at 10 µM led to a significant increase in doxorubicin uptake in IGROV1-DXR cells as compared to control cells (** $P < 0.01$; n=3) (table 1, figure 1).

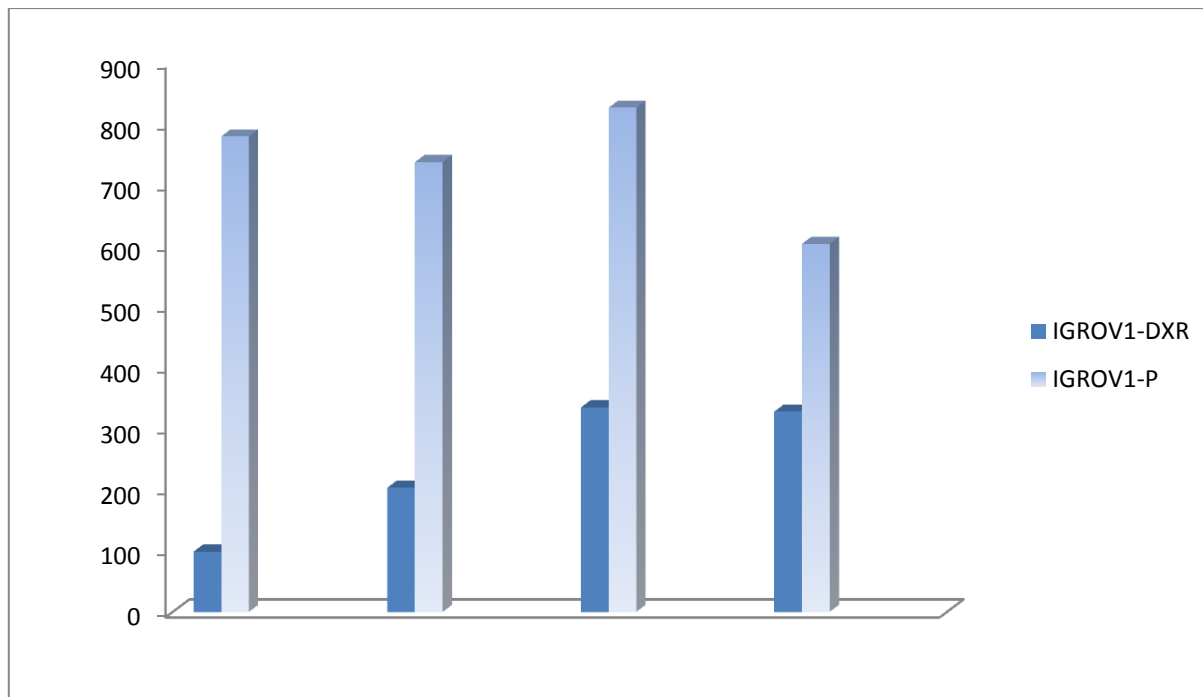
3.1.2. Effect of bevacizumab on doxorubicin intracellular accumulation

The modification of P-gp activity by bevacizumab *in vitro* was measured by the intensity of doxorubicin intracellular accumulation in IGROV1-p and IGROV1-DXR when cultured in presence of bevacizumab. Significant dose-dependent increase of doxorubicin uptake in IGROV1-DXR cells was observed when they are incubated with bevacizumab at concentrations of 1.0 µM (** $P < 0.01$; n=3) and 5.0 µM (** $P < 0.01$; n=3) as compared to IGROV1-DXR cells no treated with bevacizumab control cells. In IGROV1-p cells, a non statistically significant increase of doxorubicin uptake by cells was observed with bevacizumab as compared to control cells. Treatment by the P-gp inhibitor, verapamil, at 10 µM led to a significant increase in doxorubicin uptake in IGROV1-DXR cells as compared to control cells (** $P < 0.01$; n=3) (table 2, figure 2).

Table 1: Sorafenib pretreatment effect on doxorubicin intracellular accumulation in parental or doxorubicin resistant IGROV1 cells

Doxorubicin concentration μM (Mean \pm SD)	control	Sorafenib 5 μM	Sorafenib 10 μM	verapamil 10 μM
IGROV1-DXR	99 \pm 3	204 \pm 3*	336 \pm 18**	329 \pm 49**
IGROV1-P	780 \pm 14	738 \pm 14	827 \pm 21	604 \pm 41

* $p < 0.05$; ** $p < 0.01$



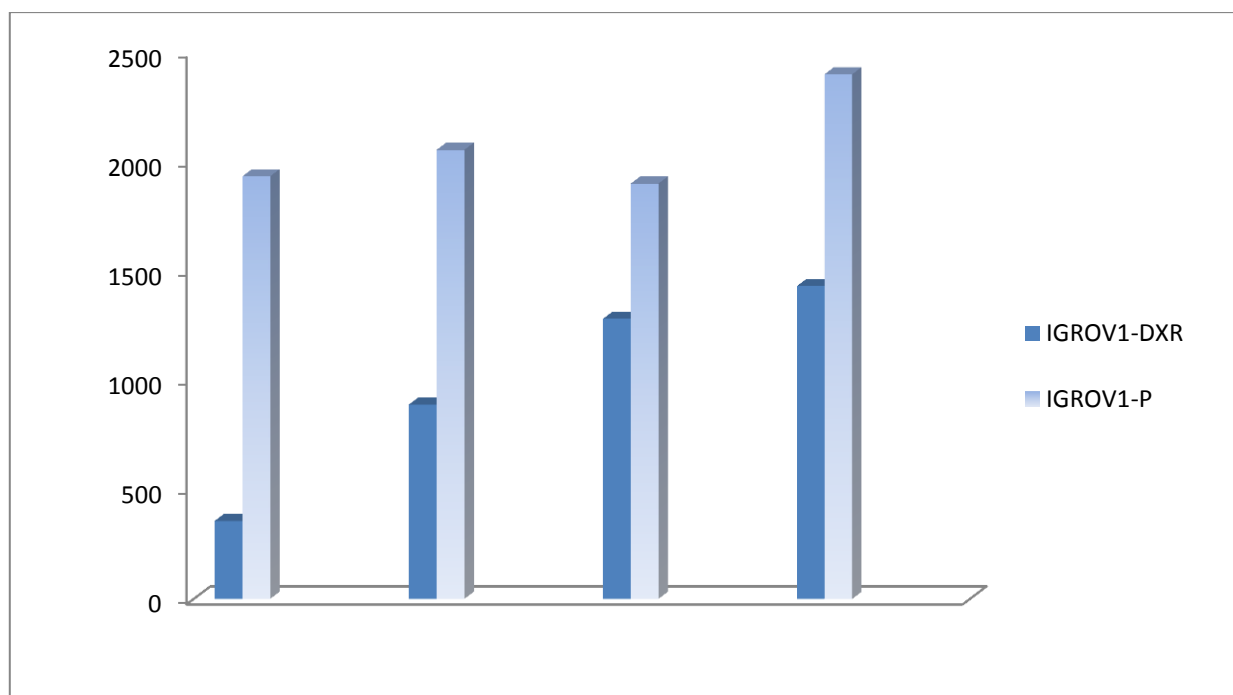
* $p < 0.05$; ** $p < 0.01$

Figure 1: Doxorubicin intracellular accumulation in IGROV1- DXR and IGROV1-P cells after preteatment with sorafenib (n=3)

Table 2: Bevacizumab pretreatment effect on doxorubicin intracellular accumulation in IGROV1 parental or doxorubicin resistant cells

Doxorubicin concentration μM (Mean \pm SD)	control	Bevacizumab 1 μM	Bevacizumab 5 μM	Verapamil 10 μM
IGROV1-DXR	357 \pm 7	890 \pm 74**	1283 \pm 24**	1432 \pm 19**
IGROV1-P	1933 \pm 62	2054 \pm 35	1900 \pm 37	2399 \pm 89

** $p < 0.01$



* $p < 0.05$; ** $p < 0.01$

Figure 2: Doxorubicin intracellular accumulation in IGROV1- DXR and IGROV1-P cells after pretreatment with bevacizumab (n=3)

3.2. Effect of bevacizumab pretreatment on plasma pharmacokinetic parameters of sorafenib in mice bearing human colorectal carcinoma xenograft

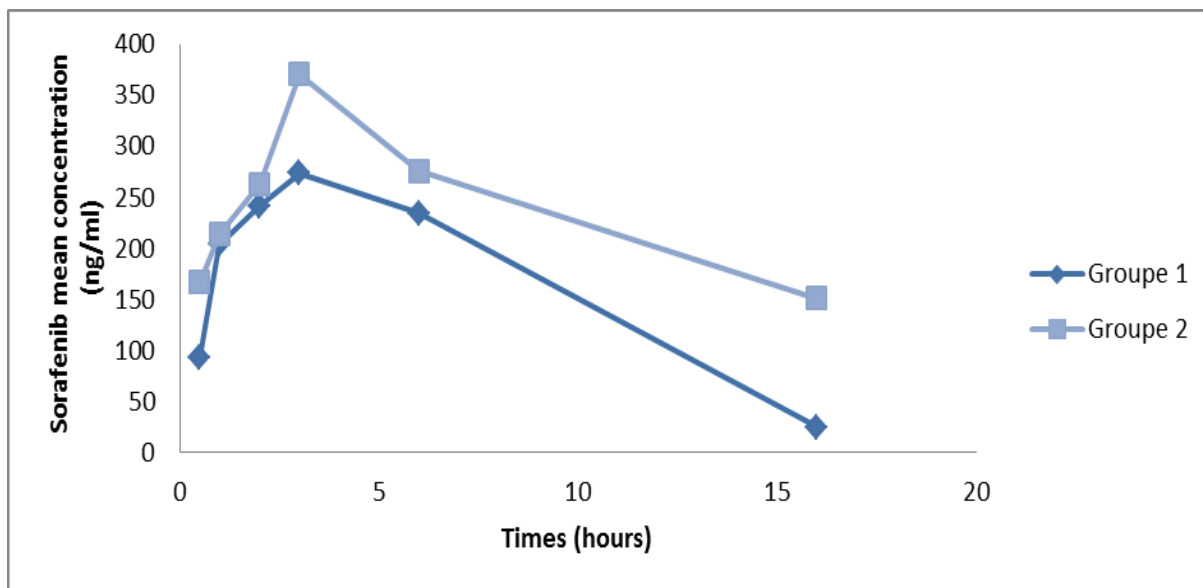
The mean plasma concentrations of sorafenib are reported in table 3 and figure 3, they represent the sorafenib plasma concentrations versus time curves. Using a noncompartmental analysis, plasma pharmacokinetic parameters of sorafenib were calculated (table II). In both non pretreated and bevacizumab pretreated groups, Tmax

was observed 3 hours after sorafenib administration, bevacizumab pretreatment does not affect the time required to reach the maximal plasma concentration of sorafenib. Sorafenib AUC was 1.44-fold higher in bevacizumab-treated group ($Z_{obs} = 2.86 > Z_{table}$), this difference was statistically significant. Cmax was 1.35 - fold higher in bevacizumab-pretreated mice group, but this increase was not statistically significant. Half-life and MRT were respectively 2.91- and 2.71 - fold higher in pretreated group.

Table 3: Plasma pharmacokinetic parameters of sorafenib after oral administration (5 mg/kg) in nude mice bearing human colorectal carcinoma and which received or not bevacizumab (5 mg/kg). A noncompartmental analysis.

	Mice that received only sorafenib	Mice that received sorafenib after bevacizumab pretreatment	Probability
Cmax (ng/ml)	273.91 ± 55.19	370.56 ± 104.75	p=0.23 (NS)
AUC 0-16h (h*ng/ml)	2640.32 ± 168.51	3795.00 ± 366.75	Z obs = 2.86>1.97 (S)
C last (ng/ml)	< LOQ	151.16 ± 3.59	
T max (h)	3	3	
t 1/2 (h)	3.57	10.39	
MRT (h)	5.84	15.84	

Cmax, mean maximal concentrations compared by student test; AUC 0-16h, Area under the curve calculated by trapezoidal method from time 0 to time 16h and compared by Bailer's method, Tmax, time to reach the maximal concentration; C last, lowest measured concentration; T 1/2, elimination half life time ; MRT, Mean Residence Time ; NS, not significant.



Groupe1: after oral administration of sorafenib (5 mg/ kg)

Groupe2: after oral administration of sorafenib (5 mg/ kg) after pretreatment with bevacizumab (5 mg/ kg)

Figure 3: Mean plasma concentration-time curve of sorafenib in the absence and in the presence of bevacizumab.

4. Discussion

The efflux of anticancer drugs from tumor cells during treatment is one of the causes of treatment failure. The expression of P-gp in the small intestine, liver and kidneys may be responsible for changes in the pharmacokinetics of P-gp-mediated drugs and then for the failure of treatment [19]. To avoid this, several P-gp inhibitors have been tested, but their use is not approved in clinical practice because of drug interactions and side effects [20].

In vitro, we observed in IGROV1-DXR cancer cells, a significant increase in the intracellular accumulation of doxorubicin in a dose dependent manner after pretreatment with bevacizumab or sorafenib. This indicates that resistance to doxorubicin was attenuated by pretreatment with either drug, due

to their modulating effect of the functionality of P-gp, an efflux pump for doxorubicin. A similar effect was observed with verapamil (a known P-gp inhibitor and positive control of our test). Bevacizumab could directly interact with P-gp and modulate its functionality.

Huang et al. have shown that expression of MDR1 was significantly decreased after sorafenib treatment in a human gastric cancer cell line SGC7901/DDP and may reverses resistance to cisplatin through down-regulating MDR1 expression [21].

Wei L. et al have explored the role of sorafenib in reversing MDR in hepatoma cells and have equally showed that sorafenib partially reversed the drug resistance of BEL-7402/FU cells to adriamycin [22].

M.R. Bani *et al.* have also shown that exposure of ovarian adenocarcinoma tumor endothelial cells to sorafenib abrogates resistance to doxorubicin and paclitaxel in vitro, increasing drug accumulation with no beneficial effect to cytotoxic drugs that are not P-glycoprotein substrate [9].

J. Dong *et al.* [8] have shown that sorafenib exerts potent inhibitory activity against epithelial-mesenchymal transition (EMT) and multidrug resistance (MDR) by inhibiting mitogen-activated protein kinase (MAPK) signaling in HCC. However, after long-term exposure to sorafenib, HCC cells exhibit EMT and resistance to sorafenib.

A meta-analysis of four studies concluded that the addition of bevacizumab to chemotherapy offers meaningful improvement in progression-free survival (PFS) and objective response rate (ORR) in ovarian cancer treatment [23].

Since sorafenib is a known substrate of P-gp, we suspected that bevacizumab could increase its concentration *in vivo*.

In our work we performed a pharmacokinetic study of sorafenib after oral administration, with or without pretreatment by bevacizumab, in plasma of mice bearing a human colorectal cancer xenograft and compared the results of both groups. T_{max} was 3 hours in both groups (pre-treated or not by bevacizumab), similar to T_{max} reported in PK studies in mice [24,25]. We observed a non-significant increase in C_{max} in the pretreated group with bevacizumab, but a significant increase in sorafenib AUC in plasma. The half-life and MRT were higher in the pretreated group with bevacizumab. These results indicate that pretreatment with bevacizumab increases plasma concentrations and exposure to the drug.

The increase of C_{max} indicates an effect on the intestinal absorption of sorafenib, the variation of which is not significant, probably due to the high bioavailability of sorafenib which could reach 80% in mice. The elimination of sorafenib is mainly biliary. In fact, the increase in the half-life of sorafenib and the MRT are signs of decreased elimination, probably due to inhibition elimination bile duct of P-gp [25].

5. Conclusion

Bevacizumab and sorafenib, two anti-angiogenic drugs could sensitize tumor cells to chemotherapies by decreasing P-glycoprotein functionalities like we shown in vitro with a beneficial effect when these drugs are combined.

Inhibition of P-glycoprotein functionality by bevacizumab could restore thus chemosensitivity and increase plasma concentration of sorafenib, probably due to a P-glycoprotein pharmacological dependent interaction.

The anti-angiogenic activity of these two drugs might also be potentiated by the modulation of P-glycoprotein functionalities that could sensitize tumor endothelial cells to cytotoxic agents.

Acknowledgement

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Compliance with Ethical Standards

The experimental protocol was approved by the Local Animal Experimentation Committee (N°26, Ministère de la Recherche et de l'Enseignement Supérieur).

Conflict of Interest

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The authors declare no conflict of interest.

Abbreviations

P-gp – P-glycoprotein
AUC – area under the curve
C_{max} – maximal concentration
T_{max} – time to reach maximal concentration
t_{1/2} – terminal half-life
CRC – colorectal cancer
MDR – Multidrug Resistance
VEGF – vascular Endothelial Growth Factor
PTX – Paclitaxel
DXR – Doxorubicin Resistant

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