Bevacizumab Modulates P-Glycoprotein Function In vitro and Increases Concentrations of Irinotecan and of its Active Metabolite SN-38 in Plasma of Human Colorectal Carcinoma-Bearing Mice

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Research Article

Abbreviations: P-gp P-Glycoprotein; AUC: Area Under The Curve; C_{max}: Maximal Concentration; T_{max}: Time To Reach Maximal Concentration; T1/2: Terminal Half-Life; CRC: Colorectal Cancer; MDR: Multidrug Resistance; VEGF: Vascular Endothelial Growth Factor; PTX: Paclitaxel; DXR: Doxorubicin Resistant

ABSTRACT

The overexpression of P-glycoprotein (P-gp) (ABCB1) is associated with multidrug resistance. Since irinotecan and its active metabolite are P-gp substrates, we tested whether bevacizumab, a monoclonal antibody directed toward VEGF (Vascular Endothelial Growth Factor), could modulate P-gp function. The first objective of this study was to evaluate whether bevacizumab could increase intracellular concentration of doxorubicin (P-gp substrate) by interacting with P-gp. The second objective was to document whether bevacizumab could modify irinotecan disposition in mice. Therefore, concentrations of irinotecan and its active metabolite SN-38 were measured by HPLC in plasma of nude mice and in plasma and tumor of mice bearing a human colorectal carcinoma xenograft when irinotecan is given orally (40 mg/kg) on day 3, alone or after a pretreatment with bevacizumab (5 mg/kg IP) on days 1 and 3.

For *in vitro* studies, two human ovarian carcinoma cells (IGROV1) overexpressing or weakly expressing P-gp were used. Bevacizumab effect on P-gp functionality was evaluated by measuring doxorubicin (P-gp fluorescent substrate) intracellular accumulation. Bevacizumab capacity to increase cytotoxicity was evaluated by MTT test. Exposure to bevacizumab with doxorubicin leads to a significant doxorubicin accumulation and a reversion of doxorubicin resistance in P-gp expressing cell lines. Pharmacokinetic analysis showed a significant increase (1.7 fold) of irinotecan AUC and C_{max} (2 fold) in plasma after pretreatment with bevacizumab in human colorectal xenograft bearing mice. A non-significant increase in irinotecan tumors AUC (1.3 fold) and C_{max} (1.3 fold) and SN-38 AUC (1.4 fold) was observed in the bevacizumab treated group. A significant trough concentration of plasma SN-38 (3.9 times higher) in bevacizumab treated nude mice was also observed. Bevacizumab increases a P-gp substrate intracellular accumulation in cell lines suggesting that bevacizumab could influence irinotecan pharmacokinetic partly due to modulation of P-gp fonction.

INTRODUCTION

The introduction of new chemotherapeutic agents combined with targeted therapies for the treatment of cancers, such as bevacizumab (AVASTIN®) and cetuximab (ERBITUX®) lead to an increase in survival [1]. Bevacizumab, a humanized monoclonal antibody binds to vascular endothelial growth factor (VEGF), the key driver of vasculogenesis and angiogenesis, and thereby inhibits the binding of VEGF to its receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2), on the surface of endothelial cells. Neutralizing the biological activity of VEGF leads to regression of the tumor vascularization, normalizes remaining tumor vasculature, and inhibits formation of new tumor vasculature, thereby inhibiting tumor growth.

However, intrinsic and acquired resistances are often observed, and acquisition of 'multi-drug resistance' (MDR) eventually causes failure of the systemic cancer treatment. Many tumors overexpress the multidrug resistance (MDR)-related efflux pump P-glycoprotein, thereby strongly reducing the potency of P-glycoprotein substrates.

Active efflux of chemotherapeutic agents from tumor cells by transmembrane pumps of the ATP-binding cassette (ABC) family has been extensively reported as a potential clinically relevant mechanism of MDR [2]. P-glycoprotein (P-gp) has a major role in MDR. Irinotecan and SN-38 are substrates of P-gp, which can cause decreased intracellular levels of these drugs.

Tumor invasion/metastasis and multidrug resistance (MDR) are the main causes of treatment failure and high mortality in all kinds of cancer patients. In a previous study [3], we showed that cetuximab, a monoclonal antibody directed towards epidermal growth factor receptor, could modulate P-glycoprotein (P-gp) functionality, and leads to an increased P-gp substrate intracellular accumulation of adriamycine in a cancerous line over expressing P-gp and also a significant increase (1.7-fold) in SN-38 disposition in plasma and in a tumor model of xenograft colorectal tumor after pretreatment with cetuximab. Wang et al. [4] have confirmed that cetuximab enhanced the efficacy of chemotherapeutic agent in ABCB1 glycoprotein overexpressing cancer cells.

Li L. et al. [5] showed synergistic enhancing effects between MDR1/P-gp and VEGF vascular endothelial growth factor (VEGF) on human laryngeal carcinoma HEP2T cell invasion *in vitro*. Moreover, VEGF level was related to P-gp expression in K562/A02 cancer cells: the action of arsenic trioxide contributed to resistance reversion of those cells by decreasing P-gp expression [6].

In addition, Broggini-Tenzer A. et al. [7] have shown that bevacizumab regimens resensitized P-glycoprotein overexpression SW480-derived tumor xenografts to microtubule-stabilizing agents like paclitaxel thus supporting this previous clinical study.

We study here the effects of bevacizumab pretreatment on P-gp functionality, favoring a decrease in the efflux of co-administered P-gp substrates.

In order to achieve our objectives, we used *in vitro* one of our models of human ovarian cancer cell lines (IGROV1), resistant to doxorubicin, because of a high P-gp efflux.

We also carried out *in vivo* a comparative pharmacokinetic study of irinotecan and its active metabolite (SN38) in plasma and in tumors in colorectal tumorgraft bearing nude mice, whether or not pretreated with bevacizumab

MATERIALS AND METHODS

Drugs

Doxorubicin was purchased from Teva (Paris France), verapamil from Abbott (Paris France). Irinotecan (CAMPTO®) [40 mg/2 mL] and bevacizumab (AVASTIN®) [25 mg/mL] were purchased from Pfizer (Montrouge, France) and Roche Registration Limited (Welwyn Garden City, United Kingdom) laboratories, respectively. Chemicals Dullbecco's modified Eagle's medium (DMEM), fœtal calf serum (FCS), phosphate-buffered saline (PBS), penicillin, streptomycin, zeocin, hygromycin B. were purchased from Gibco Invitrogen (Cergy-Pontoise France).

Irinotecan (purity C 99%) and SN-38 (purity C 98%) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). 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).

Cell Lines

The IGROV1 cells were obtained from Dr. Bénard and the CNRS 8126 laboratory at Villejuif, France.

Establishment of sensitive and resistant doxorubicin cell lines: parental drug-sensitive cell line (IGROV1-p), doxorubicin-resistant cell line (IGROV1-DXR)

The 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 [8,9]. The IGROV1-DXR cells, which overexpressed P-gp, were selected from IGROV1-p cells by continuous exposure of these cells to increased 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% CO_2 in air. IGROV1-DXR cells were maintained in medium

supplemented with doxorubicin (0.1 µg/ml).

Doxorubicin accumulation assay measured by flow cytometric analysis (FACS)

P-gp function assessment: Cell-based doxorubicin transport assays were performed with 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 the cells dissociated with 0.05% Trypsin-EDTA buffer. Cells were re-suspended in DMEM plus 10% FCS and centrifuged. After washing three times with PBS, to eliminate extracellular doxorubicin fluorescence due to doxorubicin accumulation in the cells was measured by flow cytometry analysis (acquisition of data for 10,000 cells in channel 2 (FL2). Results are expressed as total events.

Effect of bevacizumab on P-Gp Function in IGROV1-DXR lines

Effect of bevacizumab was tested at 2 different concentrations (1 mg/ml and 5 mg/ml) according to the following protocol 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 at 10 µM. P-gp function assessed by doxorubicin accumulation was expressed as percentage of doxorubicine accumulation through the following equation.

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

MTT viability test

To measure   bevacizumab cytotoxicity on IGROV1 cells, cell proliferation was determined by MTT assay. IGR-OV1 cells were plated at 20000 cells/well in 96-well plates and incubated, on attachment, in 200 µL medium with bevacizumab.

Two types of toxicity assay were performed: short-term (incubation during 2 hr, n=5) and long-term (incubation during 24 and 48 hours, n=8) of bevacizumab, at the concentrations of 0.0125 mg/ml, 0.5 mg/ml, 2.5 mg/ml, 5.0 mg/ml and 10.0 mg/ml in 10% FCS medium.

The tetrazolium dye MTT (bromure de 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium)(Sigma-Aldrich Chemical Co, Saint-Quentin, France) assay was used to evaluate the cytotoxicity of different drug concentrations, according to the procedure described by Mosman [10]. MTT test was performed with IGROV1-p and IGROV-DXR. Cells were treated with the detergent Triton X-100 1% in phosphate buffered saline (PBS) as the positive control for each set of experiments.

After bevacizumab incubation, cells were washed with PBS and MTT were added at the concentration of   0.5 mg/ml. After 4-hour incubation, formazan extraction was performed and the quantity was measured colorimetrically with ELISA at 570 nm using a multiwall-scanning spectrofluorometer (MRX II microplate reader, Dynex Technologies). Percentage of surviving of cells treated or not with bevacizumab was normalized to control, using the formula:

Cell viability (%)=[(Abs (sample) - Abs (pos. ctrl))/(Abs (neg. ctrl) - Abs (pos. ctrl))] × 100

The experiments were performed in triplicate.

Human colorectal carcinoma xenograft bearing mice model

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

The CR-IGRO16P tissue was cut into small pieces. These tissues were subcutaneously implanted into female Foxn1nu CD-1 nude mice mouse 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 tumor 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. 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).

Irinotecan and SN-38 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). 48 mice formed two groups, healthy mice and human colorectal carcinoma (CR-IGR016P) tumor bearing mice. Water and food were available ad libitum throughout the studies. The 48 nude mice received irinotecan (40 mg/kg orally on day 3) either alone or after 5 mg/kg intraperitonealy bevacizumab on days 1 and 3. At the end of the experiments, animals were anaesthetized with isoflurane, blood was collected by cardiac puncture and mice were sacrificed by cervical dislocation. Tumors were removed from the surrounding conjunctive tissue, snap frozen in liquid nitrogen and stored at - 80°C.

For irinotecan and SN-38 assay, 400 µl blood were collected in heparinized tubes at 0.25, 0.5, 1, 2, 3, 4 and 8 h after irinotecan administration, three mice per point. Blood samples were centrifuged for 10 min at 6200 g. Plasmas were harvested into clean tubes and immediately analyzed.

The same experiment was done with human colorectal carcinoma bearing mice.

Irinotecan and SN-38 plasma concentrations were measured using HPLC coupled to a fluorescence detector. For analysis, 100 µl of plasma were mixed with 200 µl of acetonitrile acidified with acetic acid [20:1 (v/v)]. The mixture was vortexed for 10 sec and then centrifuged for 10 min at 5000 rpm at room temperature. The supernatant was evaporated under a gentle stream of nitrogen at 30°C. The residue was reconstituted in 200 µl of a 0.0025 M tetra butyl ammonium/acetonitrile/trifluoroacetic acid mixture [233:100:1 (v/v/v)] and vortexed for 10 sec. An aliquot of 40 µl was then injected into the chromatographic system.

Irinotecan or SN-38 was measured using a Nucleosil C18 column (4.6 × 125 mm, 3 µm) (Interchim, Montluçon, France). The mobile phase was constituted of tetra-butyl-ammonium and trifluoroacetic acid [40000:1 (v/v)], acetonitrile and methanol [73:22:5 (v/v/v)] delivered at a flow rate of 1.2 ml/min. The eluent was monitored at 260 nm.

Irinotecan and SN-38 standard curves were correctly described by unweighted least-square linear regression. Over the irinotecan (or SN-38) plasma concentration range of 10–2500 ng/mL (5–1250 ng/mL), the determination coefficient (R^2) of the calibration curves remained >0.99. Based on quality control samples, the overall relative SD (an index of precision) was less than 12%. The irinotecan and SN-38 lower limits of quantification were 10 and 5 ng/mL. Three irinotecan and SN-38 quality controls were prepared: low (30 and 15 ng/mL), medium (750 and 375 ng/mL) and high (2000 and 1000 ng/mL).

Irinotecan and SN-38 concentrations in tumor were measured using the same HPLC/UV method described above. For analysis, the tumor was cut into small pieces of approximately 25 mm3 and then mixed with 100 µl of acetonitrile/water mixture [1:1 (v/v)]. Irinotecan and SN-38 were then extracted as described above.

Calibration standards of irinotecan and SN-38 were prepared in drug-free tumors by spiking with concentrated standards to obtain a concentration range of irinotecan (or SN-38) between 0.5 and 50 ng/g (0.25–25 ng/g). Three irinotecan and SN-38 quality controls were prepared in drug-free tumors by spiking concentrated standards: low (1.5 and 0.75 ng/g), medium (7.5 and 3.75 ng/g) and high (40 and 20 ng/g). Irinotecan and SN-38 lower limit of quantification were 0.5 and 0.25 ng/g.

Data Analysis

Since each animal provided only one sample of blood, data from animals of the same group were pooled using a naïve averaging data approach [11].

Data were analyzed separately for each treatment, giving the average concentration value of plasma and tumor. The non-compartmental analysis was performed using WinNonline 5.2 software (Pharsight, Mountain View, CA). Plasma and tumor irinotecan and SN-38 concentrations versus time curves were used to determine mean maximum concentration (C_{max}), time to achieve maximum concentration (t_{max}), mean residence time extrapolated to infinity (MRT0–¥), area under the concentration time-curve extrapolated to infinity (AUC0–¥), area under the first moment curve extrapolated to infinity (AUMC0–¥), terminal half-life (t1/2), apparent total body clearance (Clapp), apparent volume of distribution (Vapp) and trough concentration (Clast). Amount of irinotecan and SN-38 in tumor are expressed for 100 mg of tumoral tissue.

The mean areas under the concentration-time curves (AUC) were calculated by the trapezoidal method from 0 to the last concentration-time point. Irinotecan and SN-38 trough concentrations were defined as the last quantifiable concentrations.

Irinotecan and SN-38 maximal and trough concentrations were compared using a Student's t-test with a significant level at 0.05. AUC (Tlast) of the two groups (irinotecan or SN-38 + bevacizumab versus irinotecan or SN-38 alone) were compared using Bailer's method [12]; the null hypothesis is rejected if │Zobs│ is greater than 1.96. As T_{max} and half-lives are unique values, their variation after bevacizumab pretreatment was described but statistical test could not be carried out.

RESULTS

MTT Viability Test

The effect of bevacizumab on IGROV1 cell proliferation was quantified with MTT assay to determine the number of viable cells. When IGROV1 are incubated with bevacizumab during a short-time period (2 hour) in serum-supplemented media, no statistically significant difference in cell viability was observed.

A statistically significant decrease in IGROV1 cell number was observed at a concentration of bevacizumab of 10.0 mg/ml (P<0.001) in cells with 10% FCS incubated 24 hours with bevacizumab ; in serum-free conditions, there was a significant reduction in IGROV1 cell number at a concentration of bevacizumab of 2.5 mg/ml to 10.0 mg/ml (P<0.05).

When bevacizumab is incubated 48 hours with IGROV1 cells, a statistically significant decrease in IGROV1 cell number was observed at a concentration of bevacizumab of 10.0 mg/ml (P<0.001) in cells with 10% FCS incubated 48 hours with bevacizumab; in serum-free conditions, there was a significant reduction in IGROV1 cell number at a concentration of bevacizumab of

2.5 mg/ml to 10.0 mg/ml (P<0.001). The concentrations of bevacizumab required to induce cell death were higher in serum-containing conditions than in serum-free conditions because VEGF in serum binds to bevacizumab and inactivates it.

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 cells when cultured in presence of bevacizumab. Significant dose-dependent increase of doxorubicin uptake in IGROV1- DXR cells was observed when they were incubated with bevacizumab at concentrations of 1.0 mg/ml (***P<0.001; n=3) and 5.0 mg/ml (***P<0.001; n=3) as compared to IGROV1-DXR cells non treated with bevacizumab control cells. In IGROV1-p cells, a slight increase of doxorubicin uptake by cells was observed with bevacizumab at the concentration of 1.0 mg/ml (*P<0.05; n=3) while no difference was observed at 5.0 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 and IGROV1-p cells as compared to control cells (***P<0.001; n=3) **(Figure 1 and Table 1)**.

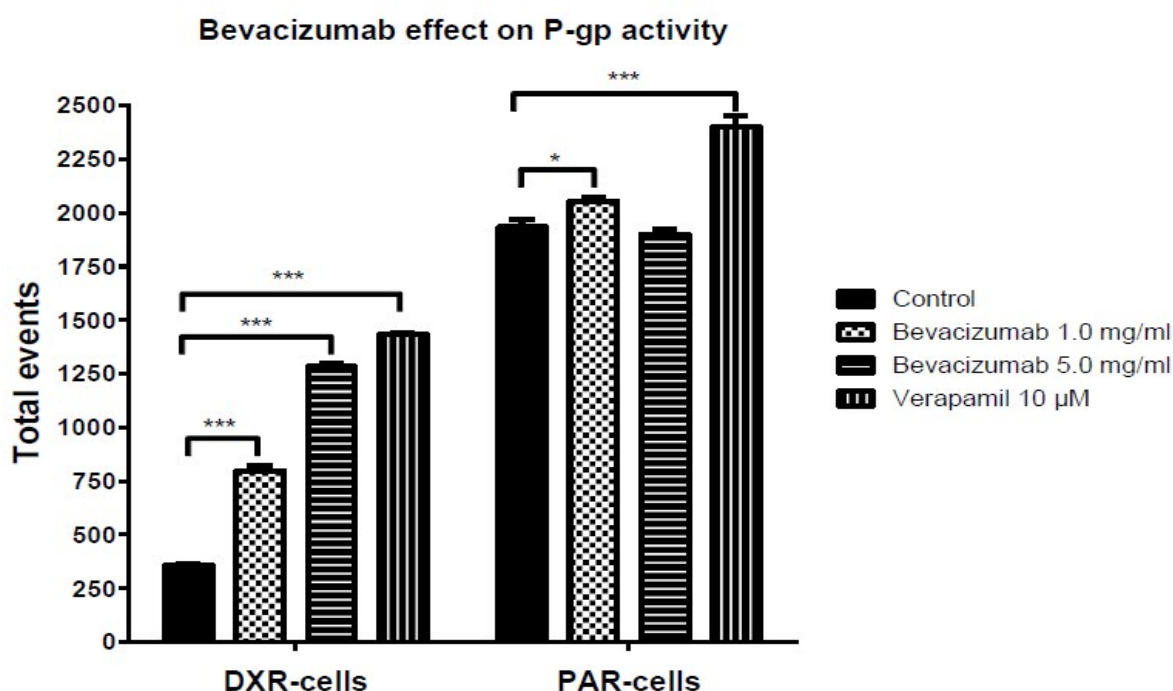**Figure 1**. Doxorubicin accumulation in IGROV1-DXR cells when cultured in presence of bevacizumab (at 1 and 5 mg/ml) (n=3) *p<0.05; ***p<0.001

Table 1 Doxorubicin accumulation in IGROV1-DXR cells when cultured in presence of bevacizumab (at 1 mg/ml and 5 mg/ml) (n=3).

	Control	Bevacizumab 1 mg /ml	Bevacizumab 5 mg /ml	Verapamil 10 μM
PAR-cells Doxorubicin Acumulation (mean ± SD)	1933 ± 62 (n=3)	2054 ± 35* (n=3)	1900 ± 37 (n=3)	2399 ± 9*** (n=3)
DXR-cells Doxorubucin Acumulation (mean ± SD)	357 ± 7 (n=3)	890 ± 74*** (n=3)	1283 ± 24*** (n=3)	1432 ± 9*** (n=3)

*p<0.05; ***p<0.001
PAR-cells=Parental drug-sensitive cell lines
DXR-cells=Doxorubicin-resistant cell lines

Effect of Bevacizumab Pretreatment on Plasma Pharmacokinetics Parameters of Irinotecan and SN-38 in Nude Mice

In **Tables 2 and 3** are reported plasma pharmacokinetics parameters of irinotecan and of SN-38.

Irinotecan maximum concentration (C_{max}) was observed at T_{max}=1h after administration.

For both groups, bevacizumab did not affect the time required to reach the maximum plasma concentration. Irinotecan AUC

was 1.12-fold lower in the bevacizumab-treated mice group; although the difference was not statistically significant.

Irinotecan terminal half-live was 1.11-fold longer in bevacizumab-treated mice group, this increase was not statistically significant.

SN-38 C_{max} value was observed at 0.5 h and 0.25 h respectively in the irinotecan alone treated group and in the bevacizumab-pretreated group.

SN-38 C_{max} was higher in bevacizumab-pretreated group. Bevacizumab pretreatment affected T_{max} and C_{max}, but not statistically significant. AUC was not modified and terminal half-live was 1.71-fold longer in bevacizumab-treated mice group.

Trough concentration of SN-38 was 3.9 times higher in bevacizumab-pretreated group than control group (p < 0.05).

Table 2. Effect of bevacizumab (5 mg/kg) pretreatment on plasma pharmacokinetics parameters of irinotecan (40 mg/kg) in nude mice (noncompartimental analysis).

Variables	Mice received irinotecan alone	Mice received irinotecan and bevacizumab	Probability
C_{max} (ng/mL) ± SD	653 ± 183	660 ± 199	NS
T_{max} (h)	1	1	
AUC (ng*h/mL) ± SD	1716 ± 162.7	1527 ± 852.7	NS (Z=0.46<1.96)
Ratio AUC	0.89		
Trough concentration (ng/mL) ± SD	6 ± 0.34	12.3 ± 4.79	NS
Half-life (h)	1.14	1.26	

Table 3. Effect of bevacizumab (5 mg/kg) pretreatment on plasma pharmacokinetics parameters of SN - 38 after irinotecan (40 mg/kg) administration in nude mice (noncompartimental analysis).

Variables	Mice received irinotecan alone	Mice received irinotecan and bevacizumab	Probability
C_{max} (ng/mL) ± SD	626 ± 172	844 ± 175	NS
T_{max} (h)	0.5	0.25	
AUC (ng*h/mL) ± SD	1286 ± 69.8	1257 ± 120	NS (Z=0.26<1.96)
Ratio AUC	0.98		
Trough concentration (ng/mL) ± SD	3.7 ± 1.4	14.5 ± 5.8	S (p=0.03 <0.05)
Half-life (h)	0.91	1.59	

Bevacizumab administration modifies irinotecan plasma and tumor pharmacokinetic parameters in mice bearing human colorectal carcinoma xenograft.

In plasma

In **Tables 4 and 5** are reported plasma pharmacokinetic parameters of irinotecan and of SN-38.

Irinotecan T_{max} was observed 1h after its administration in both groups; bevacizumab does not affect the time required for the plasma concentration to reach its maximal values.

Interestingly, irinotecan, C_{max} was 2-fold higher in bevacizumab-pretreated mice group, than in control group, this increase was statistically significant (p= 0.04). Irinotecan AUC was also 1.74-fold statistically higher in bevacizumab-treated group (Zobs= 2.54 > Z **(Table 4).**

Half-life was lower in bevacizumab-pretreated group although not significantly.

SN-38 T_{max} was observed 1h after administration of irinotecan. Regarding SN-38 C_{max} and AUC no statistically significant difference was found between both groups.

Table 4. Effect of bevacizumab (5 mg/kg) pretreatment on plasma pharmacokinetics parameters of irinotecan (40 mg/kg) in nude mice bearing human colorectal carcinoma (noncompartimental analysis).

Variables	Mice received irinotecan alone	Mice received irinotecan and bevacizumab	Probability
C_{max} (ng/mL) ± SD	855 ± 437	1706 ± 217	S (p=0.04<0.05)
T_{max} (h)	1	1	
AUC (ng*h/mL) ± SD	1908 ± 415	3323 ± 1218.4	S (Z=2.54>1.96)
Ratio AUC	1.74		

Trough concentration (ng/mL) ± SD	63.5 ± 58.1	8.4 ± 3.2	NS
Half-life (h)	3.87	0.88	

Table 5. Effect of bevacizumab (5 mg/kg) pretreatment on plasma pharmacokinetics parameters of SN - 38 after irinotecan (40 mg/kg) administration in nude mice bearing human colorectal carcinoma (noncompartimental analysis).

Variables	Mice received irinotecan alone	Mice received irinotecan and bevacizumab	Probability
C_{max} (ng/mL) ± SD	596 ± 179	749 ± 472	NS
T_{max} (h)	1	1	
AUC (ng*h/mL) ± SD	1109 ± 82.8	1154 ± 102.7	NS (Z=0.21<1.96)
Ratio AUC		0.96	
Trough concentration (ng/mL) ± SD	14.6 ± 6.8	30.3 ± 19.04	NS
Half-life (h)	2.62	1.35	

In tumor

In **Tables 6 and 7** are reported tumor pharmacokinetic parameters of irinotecan and of SN-38.

Irinotecan C_{max} and AUC were 1.3-fold higher in bevacizumab-treated group than in the control. This difference was not statistically significant.

As shown in **Table 5,** time necessary to reach the maximal concentration was 1h shorter in the bevacizumab-pretreated mice group with higher C_{max} value, than in the irinotecan alone treated group.

SN-38 C was observed 4h after administration of irinotecan in the 2 groups but no statistically significant difference was found. Concerning SN-38 AUC in bevacizumab-treated group, it was 1.4 fold higher than in not treated group, this difference was not statistically significant.

Table 6. Effect of bevacizumab (5 mg/kg) pretreatment on tumor pharmacokinetics parameters of irinotecan (40 mg/kg) in nude mice bearing human colorectal carcinoma (non-compartimental analysis).

Variables	Mice received irinotecan alone	Mice received irinotecan and bevacizumab	Probability
C_{max} (ng/mL) ± SD	1294 ± 142	1797 ± 196	NS
T_{max} (h)	2	1	
AUC (ng*h/mL) ± SD	5982 ± 3513.9	7818 ± 468.8	NS (Z=1.14<1.96)
Ratio AUC	1.31		
Trough concentration (ng/mL) ± SD	231.2 ± 127.5	698.4 ± 305.6	NS
Half-life (h)	2.18	3.74	

Table 7. Effect of bevacizumab (5 mg/kg) pretreatment on tumor pharmacokinetics parameters of SN – 38 after irinotecan (40 mg/kg) administration in nude mice bearing human colorectal carcinoma (noncompartimental analysis).

Variables	Mice received irinotecan alone	Mice received irinotecan and bevacizumab	Probability
C_{max} (ng/mL) ± SD	252 ± 155	371 ± 121	NS
T_{max} (h)	4	4	
AUC (ng*h/mL) ± SD	1345 ± 153.5	1868 ± 224	NS (Z=1.57<1.96)
Ratio AUC	1.39		
Trough concentration (ng/mL) ± SD	110 ± 82.8	230.9 ± 93.6	NS
Half-life (h)	3.34	5.86	

Relationship between Plasma and Tumor Irinotecan Concentrations in Mice Treated by Bevacizumab

At each point, irinotecan plasma and tumor mean values of mice treated with irinotecan and bevacizumab were divided by irinotecan plasma and tumor mean values of mice treated with irinotecan alone.
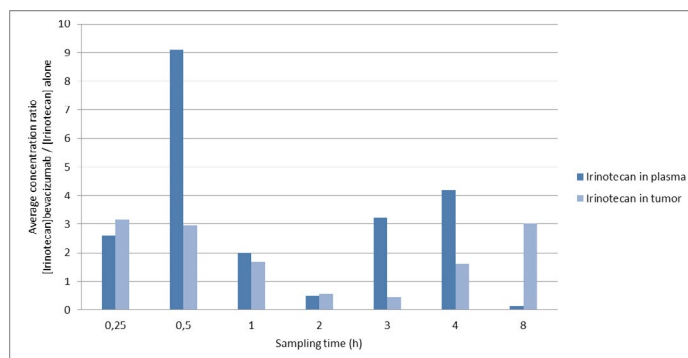
Figure 2. Mean plasma and tumor concentrations ratios of irinotecan.

In **Figure 2**, plasma value ratio of irinotecan increases at 0.5 h after administration of irinotecan and decreases at 8 h and tumor value ratio of irinotecan increase at 8 h after administration of irinotecan.

Relationship between Plasma and Tumor SN-38 Concentrations in Mice Treated By Bevacizumab

SN-38 plasma and tumor mean values of mice treated with irinotecan and bevacizumab were divided by SN-38 plasma and tumor mean values of mice treated with irinotecan alone.
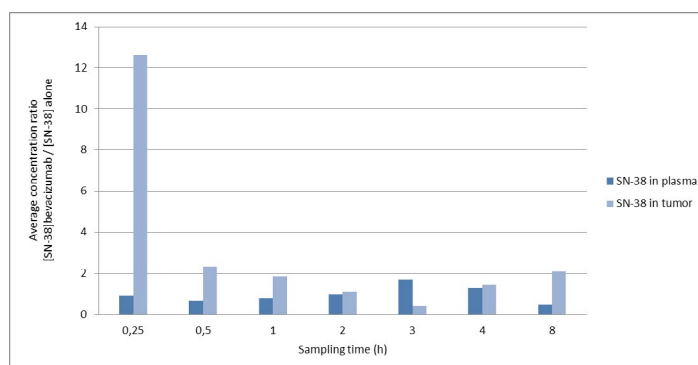

Figure 3. Mean plasma and tumor concentrations ratios of SN38.

As shown in **Figure 3**, higher SN-38 plasma value ratios were observed at two points, 3 and 4 h. Higher SN-38 tumor value ratios were observed at two points, 0.25 h and 8 h.

Effect of Bevacizumab on Irinotecan Metabolism into SN-38

SN-38 plasma and tumor mean concentrations were divided by irinotecan plasma and tumor mean concentrations in mice treated or not with bevacizumab. These plasma ratios are shown.
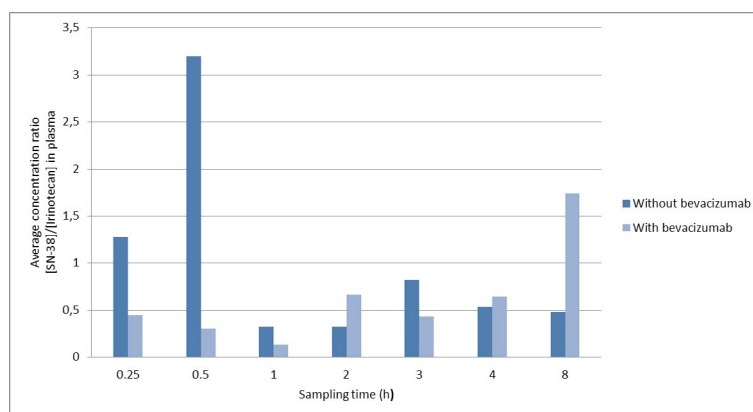

Figure 4. Ratios of SN38 mean plasma concentrations divided by irinotecan mean plasma concentrations in the absence and presence of bevacizumab.

In **Figure 4**, in plasma, higher SN-38 formation was observed in the absence of bevacizumab in the early sampling times

(0.25 and 0.5 h), and their ratios decreased in the late sampling times reflecting higher SN-38 formation in the presence of bevacizumab for the late sampling time (4 h and 8 h).
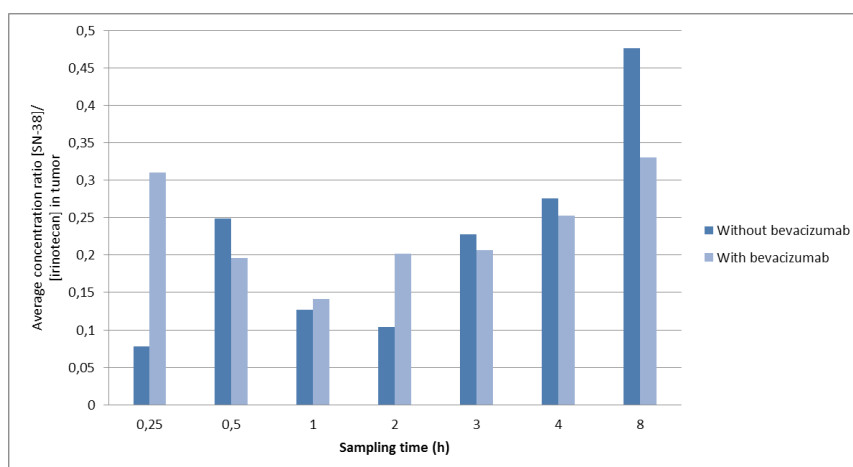
These tumor ratios are shown below.

Figure 5. Ratios of SN - 38 mean tumor concentrations divided by irinotecan mean tumor concentrations in the absence and presence of bevacizumab.

In **Figure 5**, In tumor a higher SN-38 formation was observed in the presence of bevacizumab for the times 0.25 h and 2 h in the early sampling times and their ratios were smaller in the later to compared without bevacizumab.

The effect of bevacizumab on SN-38 formation was not constant in plasma and tumor.

DISCUSSION

Chemoresistance is the main limitation to cancer treatment. Resistance to conventional drugs involves ATP binding cassette (ABC) transporters and decreases the efficacy of chemotherapeutics.

Many drugs nowadays have proven their efficiency in the treatment of cancers. However, since there is a correlation between drug level exposure, efficacy and adverse effects [13], it becomes difficult to increase drug posology without increasing side effects risk. It is thus important to study other approaches to increase efficacy by attempting to maintain the drug within the tumor cells it had entered in and by controlling its efflux. This is why it appears interesting in recent protocols to modulate anticancer activity by association with drugs meant to prevent the efflux from the cell to maintain a cellular high concentration.

One of the objectives of this study was to evaluate how a pretreatment with bevacizumab could modify *in vitro* the intracellular increase of doxorubicin concentrations as a modulation of P-gp functionality.

That for we used two cell lines derived from human ovarian carcinoma, the original line IGROV1-p (doxorubicin-sensitive) and the IGROV1-DXR (overexpressing P-gp).

In vitro we observed in IGROV1-DXR cancer cells, a significant increase of intracellular accumulation of doxorubicin (a P-glycoprotein substrate) after pretreatment with bevacizumab. This indicates that resistance to doxorubicin has been attenuated by bevacizumab due to its modulating effect of the functionality of the P-glycoprotein. A similar effect was observed with verapamil (a known inhibitor of P-gp and a positive control in our test). Bevacizumab could directly interact with P-gp and modulates its functionality. A study in breast or lung cancer xenograft bearing nude mice showed that inhibition of the biological activity of VEGF by bevacizumab can improve the absorption and efficacy of co-administered chemotherapy, thus increasing the uptake of paclitaxel (PTX) and causing its higher intra-tumor concentration of paclitaxel, leading to decreased permeability and tumor volume [14]. A case study has reported the resensitization of a paclitaxel-resistant metastatic breast cancer patient to paclitaxel therapy by addition of bevacizumab to his chemotherapy regimen [15].

Furthermore, comparable results have been observed by our team after pretreatment with cetuximab, another monoclonal antibody directly against epidermal growth factor receptor. This could partly explain higher concentration of cytotoxic agents in cancer cells, mainly those that are substrates of P-glycoprotein [16].

We performed *in vivo* a comparative pharmacokinetic study of irinotecan and of SN38 (known to be P-gp substrates [17]) in plasma and in tumors in colorectal tumor graft bearing nude mice, whether or not pretreated with bevacizumab before irinotecan administration.

We observed a significant increase of plasma pharmacokinetic parameters of irinotecan after pretreatment with bevacizum-

ab, in particular the AUC, as well as the C_{max} which was twice higher in tumor-bearing mice than in not pretreated mice. As it had been shown in previous published studies [18,19], the modulation of P-gp function will result in an increase of C_{max}, this transmembrane pump is over-expressed in individuals with colorectal cancer cells [20,21], which potentiates more the expulsion of cytotoxic agents and the decrease of their plasma concentration. This could also explain the lack of statistically significant results in nude mice perhaps because of the lower level of P-gp expression compared to tumor-bearing mice group.

In tumors, we observed a non-significant increase in irinotecan AUC and C_{max} (1.3 fold) and SN-38 AUC (1.4 fold) after a pretreatment with bevacizumab. Irinotecan AUC tumor increase, although statistically non-significant, can be due to an increase in the distribution into tissues.

Despite the lack of a statistical significant increase, it may be suggested that irinotecan and its active metabolite SN-38 were slightly better distributed in colorectal xenograft in combination with bevacizumab. In addition, T_{max} reduction may reflect an increase in tumor permeability of irinotecan. This could be explained partly by the modulation of the function of the P-gp by bevacizumab in tumor. Moreover, a study carried out in 2015 [22], on human brain xenograft-bearing mice (Glioblastoma) demonstrated that the pretreatement with bevacizumab could slightly increase the tumor penetration of temozolomide (a minor substrate of P-gp), via a mechanism involving inhibition of P-gp [22].

To document whether bevacizumab may modify irinotecan metabolism through the formation of SN-38, we evaluated the plasma and tumor average concentration ratio of mice treated by irinotecan with or without bevacizumab **(Figures 4 and 5).** This evaluation showed that in plasma the first sampling times, bevacizumab pretreatment led to a decrease in SN-38 formation while it led to its increase in the tumor (0.25 and 2 h).

The results can be explained by the fact that bevacizumab modulates P-gp functionality, facilitating irinotecan diffusion into tissues such as liver and kidneys. A higher distribution of irinotecan and SN-38 due to bevacizumab led to a second increase of concentrations of both molecules at 4 h sampling time.

The results obtained in this study suggest that irinotecan distribution is the step most affected by the pretreatment with bevacizumab. Another mechanism was mentioned in the literature referring to increase concentration of combined cytotoxic agents in cancer cells with bevacizumab. Transient compensation of angiogenesis by bevacizumab leads to an increase of vascular permeability and a reduction in tumor edema, an improvement in the distribution of the co-administrated drugs in cancer cells and a cell death increase [23,24]. In fact, a better induction of apoptosis is suggested due to the administration of bevacizumab with irinotecan, when compared to monotherapy, resulting in cell death increase among malignant tumor cells [25].

A small pharmacokinetic substudy AVF2170g of Phase III clinical trial, suggested that the addition of bevacizumab to the chemotherapy regimen bolus-IFL (irinotecan/5-FU/leucovorin) in patients with metastatic colorectal cancer did not change plasma concentrations of irinotecan [26], but change the concentrations of SN-38, on average, 33% higher in patients receiving bevacizumab in combination with bolus-IFL, when compared to patients with bolus-IFL alone.

The study performed in 2005 by Gaudreault et al. [27], on the effect of bevacizumab on pharmacokinetics of irinotecan, SN-38 and 5-FU in naïve cynomolgus monkeys receiving the Saltz regimen (irinotecan/ 5-FU/leucovorin), concluded that bevacizumab does not influence the pharmacokinetics of the associated drugs. Since this study was conducted on healthy animals, it can be considered that this conclusion is consistent with our findings concerning the lack of a pharmacokinetic interaction between the two drugs in tumor-free mice. Then, it is assumed that the presence of an advanced tumor with a surexpression of P-gp could be a necessary condition for the occurrence of modification of irinotecan and SN-38 pharmacokinetics by bevacizumab.

Nan Qi et al. [28] have showed in a patient with non–small cell lung cancer that the combined use of paclitaxel (a Pgp substrate) and bevacizumab displayed a rapid distribution of paclitaxel with an increase in its elimination half-life in the pleural fluid. Mean residence time of paclitaxel increased in the presence of bevacizumab. They assumed that this rapid distribution referred to the change in vessel permeability; since the decrease permeability would be beneficial to paclitaxel remaining the target tissues.

Our results have shown also an increased half-life elimination of irinotecan and SN38 in tumor in presence of bevacizumab

CONCLUSION

In addition to its anti-cancer activity, bevacizumab could modulate P-gp functionality and improve the efficacy of P-gp substrates drugs, such as doxorubicin. Bevacizumab significantly increased SN-38 plasma trough concentration in mice and irinotecan AUC and C_{max} plasma in mice bearing human colorectal carcinoma. This result could open new research perspectives on the role of monoclonal antibodies in the MDR phenomenon and their association with substrates drugs.

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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

The authors declare no conflict of interest.

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