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Antitumour activity of the silybin-phosphatidylcholine complex, IdB 1016, against human ovarian cancer

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Abstract

This study aimed to assess, in an *in vivo* experimental model, the growth inhibitory effects of IdB 1016 (Silipide, a complex of silybin/phosphatidylcholine) when used as a single agent against human ovarian cancer. We also wanted to investigate the mechanism of the antiangiogenic action by assessing Vascular Endothelial Growth Factor (VEGF) levels and by using macroarray technology to evaluate the regulation of a panel of genes involved in angiogenesis. We also aimed to establish the plasma and tumour bioavailability of silybin after repeated administration of IdB 1016. Female nude mice bearing human ovarian cancer xenografts (A2780) received 450 mg/kg/day IdB 1016 daily by oral gavage until the end of the study. At sacrifice, blood and tumour specimens were collected and subsequently processed for the determination of silybin levels, VEGF levels or a gene expression profile. IdB 1016 was significantly active in inhibiting ovarian tumour growth. Treatment with 450 mg/kg/day for a total of 20 administrations produced a tumour weight inhibition (TWI%) of 78% and a Log10 Cell Kill (LCK) of 1.1. Free silybin levels were found to be 7.0±5.3 μg/ml and 183.5±85.9 ng/g tissue (mean±standard deviation (S.D.)) in the plasma and tumour samples, respectively. No significant differences were found in the concentration of human VEGF in xenografts from control and IdB 1016-treated mice. The array analysis suggested the downregulation of the VEGR receptor 3 and the upregulation of angiopoietin-2 as potential mechanisms for the antiangiogenic activity. In conclusion, these findings suggest IdB 1016 is a good candidate, with a relevant clinical potential, for use in the management of recurrent ovarian cancer. A phase II, non-randomised clinical study is now ongoing in our Institute aimed at evaluating the efficacy of daily administrations of IdB 1016 in the serological recurrence of ovarian cancer.

Keywords: IdB 1016; Silybin; Silipide; Ovarian cancer; Angiogenesis

1. Introduction

Ovarian cancer is one of the most sensitive of all solid tumours to antineoplastic chemotherapy and responses are expected in 80% women who receive standard platinum- and paclitaxel-based treatment. Nevertheless, most women with advanced ovarian cancer will ultimately relapse and develop a drug-resistant disease with an overall 5-year survival of 50% [1]. Actually, it is accepted that the general intent of current treatments for patients with recurrent disease is palliative [2] and, for this reason, the development of new treatment strategies in this clinical setting is of particular interest. Importantly, the favourable toxicity profile of the proposed therapeutic regimens and the impact on the overall quality of life are regarded as important elements for the selection of new agents.

IdB 1016 (Silipide) is a complex of the flavonoid silybin with phosphatidylcholine in a molar ratio of 1:1, which, after oral administration, gives plasma levels that are significantly higher than those found after the administration of silybin or silymarin to rats [3] or to humans [4]. Silybin is a naturally occurring polyphenolic flavonoid that constitutes the major active component in silymarin, a standardised extract of the milk thistle *Silybum marianum*, that is widely used both as a drug and a dietary supplement in Europe and the United States. In previous studies, we and others have

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found silybin to be active, with growth inhibitory effects on human prostate, ovarian, breast and cervical carcinoma cells [5–10]. We have also recently demonstrated that IdB 1016 is able to potentiate the antiproliferative activity of optimal or sub-optimal doses of cisplatin against human ovarian cancer xenografted into athymic mice [11]. Other authors have also reported a synergistic interaction between silybin and other cytotoxic drugs in *in vitro* studies [12]. Finally, we showed for the first time an anti-angiogenic effect of IdB 1016 in an *in vivo* experimental model [11], in keeping with a previous report [13] which demonstrated that silymarin treatment inhibited human umbilical vein endothelial cells (HUVEC) growth, endothelial matrix metallo-proteinase (MMP-2) secretion, *in vitro* capillary tube formation, as well as the secretion of vascular endothelial growth factor (VEGF) by prostate and breast cancer cells.

The aim of the present study was to extend our previous observation regarding the antitumour activity of IdB 1016 by evaluating, in an *in vivo* model, its growth inhibitory effects when used as a single agent in human ovarian cancer cells. In this context, the bioavailability of silybin after repeated administration of pharmacologically-active doses of IdB 1016 was also confirmed by analysing both plasma and tumour levels of silybin. On the basis of observations reported from Jiang and colleagues in Ref. [13], we also looked at the potential silybin/IdB 1016 effects on VEGF production/secretion by A2780 cells, by determining protein levels in conditioned media, as well as in tumour specimens and serum. Finally, in order to elucidate the molecular mechanism responsible for the effects observed, the modulation exerted by IdB 1016 on a gene expression profile was assessed by an array analysis of vehicle and IdB 1016-treated xenografts.

2. Materials and methods

2.1. Cell line

A2780 cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). According to ECACC suggestions, cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 1% non-essential amino acids mixture and 1% kanamycin. Cells, propagated as a monolayer culture, were trypsinised twice weekly and plated at a density of 1×10^5 cells per ml. All cultures were incubated at 37 °C, under 5% CO_2, in a high humidity atmosphere.

2.2. In vivo studies

Female athymic mice (HSD: Athymic Nude-nu), 4 weeks old and within a weight range of approximately 15–20 g, were obtained from Harlan Nossan S.r.l. Correzzana (MI), Italy. Animals were housed in clear solid-bottomed polycarbonate cages with sterile sawdust bedding material, and kept in an isolator in which the controls were set to maintain temperature and relative humidity at 26±2 °C and 50%, respectively. Artificial lighting provided a 24-h cycle of 12 h of light and 12 h of dark. Sterile water and food were supplied *ad libitum*. On the day of xenograft implantation (day 1 of the study) cells, cultured as described above, were trypsinised, collected in RPMI 1640 medium without supplements, and a suspension of 8×10^6 cells was injected subcutaneously (s.c.) in the right flank of each animal (0.2 ml per mice). During the study, mice were checked daily for any adverse clinical reactions and weighed three times per week. Tumour dimensions were measured with a calliper three times per week.

IdB 1016 (INDENA, Milan, Italy) was formulated daily, directly before use, by suspending the test substance in 0.5% sodium carboxymethylcellulose (CMC) in sterile water, at a concentration of 45 mg/ml. Starting from the day of inoculation, mice received 450 mg/kg/day of IdB 1016 by oral gavage daily, 6 days per week, for a total of 20 (experiment 1) or 14 (experiments 2 and 3) administrations. The dose selection for this study was based on previous results on the *in vivo* anti-angiogenic activity of the compound [11]. Control animals received the vehicle. Experiment 1 ($n=10$ mice/group) was carried out in order to assess the efficacy of IdB 1016 in inhibiting human ovarian cancer growth when used as a single agent. Experiments 2 ($n=15$ controls and 30 treated mice) and 3 ($n=15$ mice/group) were designed to obtain at the end of the study both blood and tumour samples and were terminated earlier in order to avoid unscheduled deaths. At the end of the study, animals were deeply anaesthetised with diethyl ether and venous blood samples were collected from the caudal *vena cava,* approximately 45 min after the last administration: these samples were subsequently processed for the determination of silybin and VEGF levels (see below). All tumours were also removed, immediately placed in liquid nitrogen and stored at −80 °C for subsequent determination of tissue silybin levels, VEGF levels or for DNA array analysis.

Procedure and facilities followed the requirements of Commission Directive 86/609/EEC, concerning the protection of animals used for experimental and other scientific purposes. Italian legislation is defined in the D.L. No. 116 of 27 January 1992.

2.3. Evaluation of antitumour activity

Solid tumour weight was estimated from two dimensional measurements (mm): Tumour Weight = (Length×Width^2)/2 [14]. Efficacy was expressed as the percentage of maximum tumour weight inhibition

(TWI%). The ratio between the median tumour weight of treated tumours and that of control tumours×100 (T/C%) was assessed on each day of measurement and used to calculate the TWI%: TWI% = 100−T/C%. The highest inhibition of tumour weight was considered for each experimental group.

In addition, the tumour growth delay ($T-C$) was calculated by the difference in median time required for the treatment (T) and the control (C) group tumours to reach a pre-determined size (1000 mg). The tumour doubling time (T_d) was estimated from the best fit straight line from the log-linear plot of the control group tumours in exponential growth (100–1000 mg range). The log10 cell kill (LCK) achieved by drug treatment was calculated from the following formula:

$$\text{LCK} = \frac{T - C \text{ value in days}}{3.32 \times T_d}$$

Due to the early termination, Tumour Growth Delay and LCK were not calculated in experiments 2 and 3.

2.4. VEGF assay

VEGF levels were determined in the conditioned medium from A2780 cells (human VEGF), in tumour specimens (human VEGF) and in mouse serum samples (both mouse and human VEGF).

A2780 tumour cells were seeded at a density of 1×10^5 cells/ml in complete medium and, after exposure to 10 μM silybin or vehicle (dimethylsulphoxide (DMSO)) for 24 h, supernatants were aspirated, rendered cell-free by centrifugation and stored at −20 °C until use.

Blood samples obtained at sacrifice, were allowed to clot for 2 h at room temperature before centrifugation for 20 min at 2000*g*, at room temperature; serum was separated and frozen at −20 °C until use.

Frozen tumour tissues were homogenised in 10 mM potassium phosphate buffer, pH 7.5, containing 0.1% (v/v) monothioglycerol at 4 °C [15]. The homogenate was centrifuged at 20 000*g* for 10 min at 4 °C and supernatant collected for analysis. Protein levels in homogenate supernatant were determined using the Coomassie Brilliant blue G-250 kit from Bio Rad (Italy).

VEGF assays were performed by means of a commercial quantitative immunoassay kits for human (DVE00, Research & Diagnostic Systems, Minneapolis, MN) and mouse (MMV00 Research & Diagnostic Systems) VEGF. Analyses were carried out according to the manufacturer's protocol using VEGF standards ranging from 15.6 to 2000 pg/ml (human VEGF) and from 7.8 to 500 pg/ml (mouse VEGF). All samples were analysed in duplicate in the same assay. The maximal allowed sample duplicate error was 10%; duplicates falling outside of this error margin were re-analysed.

2.5. Plasma and tissue determination of the silybin level

Free silybin levels were determined by high performance liquid chromatographic (HPLC) analysis in both the plasma and tumour samples. Analytical procedures were based on the method previously described by Martinelli and colleagues in Ref. [16]. Briefly, blood samples were collected into heparinised tubes and centrifuged as soon as possible after collection, for 20 min, at 2500*g* and +4 °C. Plasma obtained was immediately frozen and stored at −20 °C until use. Tumour samples were homogenised in citrate buffer, pH 4, and subsequently centrifuged for 10 min at 2160*g* and +4 °C. Supernatant was collected and stored frozen until use. In order to determine the free silybin levels, both plasma and tumours samples, prepared as described above, were added to citrate buffer, pH 4, loaded onto Extrelut 3 columns and eluted twice with *tert*-butylmethylether. After drying the eluates under a stream of nitrogen at 45 °C, the residues were reconstituted with an aliquot of the mobile phase (see below) and used for HPLC analysis. Extracted samples were loaded into a LiChrosorb Diol 5-μm column (12.5×4.0 mm) and eluted using, as mobile phase, a solution of *n*-hexane/ethanol (70:30 v/v) containing 0.2% phosphoric acid; the flow rate was 0.7 ml/min and the detection was performed at 214 nm. Silybin concentration was quantified by comparing peak areas with standard curves, prepared by adding known increasing amounts of silybin to pooled plasma and processed as described above. The liquid chromatograph consisted of a model 2290 Waters Alliance equipped with a model 486 variable-wavelength absorbance ultraviolet-visible (UV-VIS) detector (Waters Assoc., Milford, MA, USA) and a LiChrosorb Diol 5-μm chromatographic column (Hichrom Limited, Theale Berkshire, UK). The analytical procedure was performed by Analyst srl (Trezzano sul Naviglio, Milan, Italy).

2.6. Array analysis

2.6.1. RNA extraction

Frozen xenografts were homogenised in Trireagent (Molecular Research Center Inc., Cincinnati, OH, USA) and total RNA was purified according to the manufacturer's protocol. After spectrophotometric quantitation, total RNA was checked for quality in an agarose gel and then messenger RNA (mRNA) was isolated using an Oligotex resin (Qiagen, MI, Italy).

2.6.2. Probe synthesis

500 ng of mRNA and 4 μl of Panorama Human Cytochine primer mix (Sigma, St. Louis, MO, USA) were annealed following the manufacturer's instructions and labelling was performed with 50 units of AMV reverse transcriptase (Sigma) and 2 μl of [α-^{33}P]dCTP

(10 mCi/ml; 3000 Ci/mmol specific activity). Labelled cDNA was purified by column chromatography.

2.6.3. Hybridisation of cDNA probed to the array and array analysis

CDNA probes were heat-denatured and then chilled on ice. Panorama Human Cytokine Gene Array (Sigma) is a positively-charged nylon membrane (8×12 cm) spotted with cDNA fragments representing 838 known genes encoding cytokines, chemokines and other immunomodulatory factors, and their receptors. Nine housekeeping genes and genomic sequences are also present on the array. Arrays were prehybridised at 65 °C for 1 h with 5 ml of Panorama Hybridisation solution and 500 μg of heat-denatured, sheared salmon sperm DNA; hybridisation was performed for 18 h at 65 °C in roller bottles, with continuous agitation. Hybridised arrays were washed and exposed in a Thyphoon 8600 phosphoimager (Amersham Biosciences, Amersham, UK). Images were saved in TIFF format and quantified using the scanalyze software package. A cut-off of 2-fold up- or downregulation was used to define differentially-regulated genes. Thereafter, the data obtained were transformed into base 2 log and the ratio IdB 1016 treatment versus control was calculated as previously described in Ref. [17]. Three biologically-independent experiments were performed. Results were then averaged and genes that were differentially regulated, when the mean of the three independent experiments was calculated, were selected.

2.7. Statistics

To detect possible significant differences induced by IdB 1016 on VEGF production/secretion, in tumour as well as serum samples, the Student's *t*-test was used. *P* values were considered to be significant when they were less than 0.05.

3. Results

3.1. Antitumour activity

Results obtained in this series of preclinical studies showed that IdB 1016 is significantly active in inhibiting tumour growth when given to athymic mice bearing human ovarian cancer cells (A2780) (Fig. 1). Treatment with 450 mg/kg/day for a total of 20 days (total dose 9000 mg/kg, experiment 1) produced a tumour weight

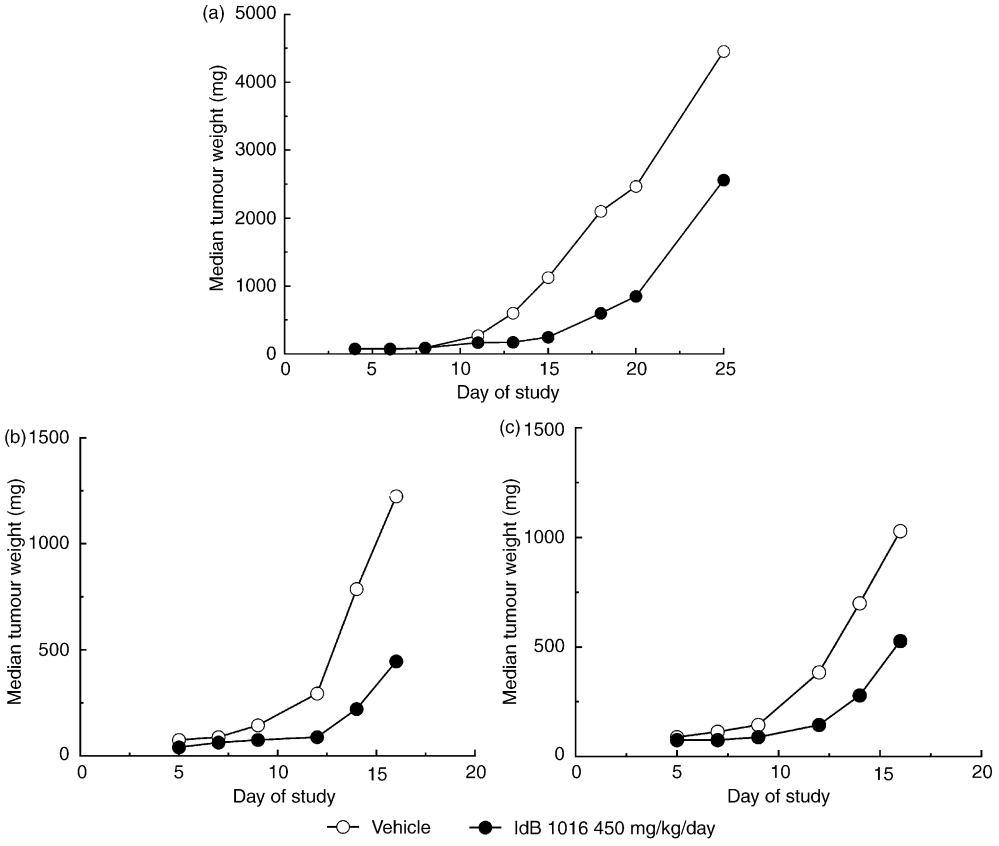

Fig. 1. Line plot charts showing the growth of A2780 xenografts in athymic mice receiving vehicle or 450 mg/kg/day IdB 1016, 6 days/week, for a total of 20 (a) or 14 administrations (b and c). The median tumour weight of 10–30 mice per group is depicted (see Materials and methods for details).

inhibition of 78% (day 15 of the study) and a LCK of 1.1. Comparable results were obtained in experiments 2 and 3, where tumour-bearing animals received the same daily doses of the drug for a total of 14 days (total dose 6300 mg/kg). TWI%s of 63 and 72%, respectively, were achieved in studies 2 and 3, on days 12 and 14, respectively; due to the early termination, LCK values were not calculated. Importantly, no signs of apparent toxicity, such as body weight loss or reduced food consumption, were shown by the treated animals throughout the studies.

3.2. Silybin bioavailability

Silybin levels were determined by HPLC analysis in both the plasma and tumour samples. The free silybin plasma level was 7.0±5.3 μg/ml (mean±standard deviation (S.D.)); the silybin concentration in the tumour samples was 183.5±85.9 ng/g tissue (mean±S.D.).

3.3. Serum VEGF

Data obtained in the present study showed that human VEGF (hVEGF) is actively secreted by the A2780 ovarian cancer cell line. However, no differences were seen in hVEGF levels in the conditioned medium from vehicle-and silybin-treated cells, values being 1590±69 and 1584±96 pg/ml, respectively (mean±standard error of the mean (SEM) of three different experiments). Consistently, hVEGF concentrations determined in the tumour specimens from control and IdB 1016-treated mice were found to be comparable, with values of 59±11 and 43±15 pg/mg protein, respectively. Values equal to or below the minimum detectable levels were found in both the control and IdB 1016-treated groups when mouse serum was analysed for hVEGF levels (data not shown). Finally, determination of mVEGF in serum samples from animals bearing A2780 xenografts did not show any significant differences attributable to IdB 1016 treatment: mean values were 118±16 and 165±27 pg/ml in the control and treated animals, respectively.

3.4. Array analysis

The gene expression profile of tumour xenografts from both control and IdB 1016-treated animals was assessed in three different experiments by using a cDNA macroarray containing 838 genes. Twenty-two of 838 represented were not expressed in the A2780 cells. Of the remaining 816 genes, 15 (1.8%) and 17 (2.1%) genes exhibited downregulation and upregulation upon IdB 1016 treatment, respectively (in three independent experiments). The results are summarised in Fig. 2 and in Table 1. Most of the genes found to be downregulated are involved in the control of the immune and monocyte-macrophage systems (Toll-like receptor 1 (*TLR1*), IL-12 subunit p35, Granulocyte-Macrophage Colony-Stimulating Factor (*GM-CSF*), chemokine *MIP-1alpha* (Macrophage Inflammatory Protein 1-alpha), *IL-11*, defensin A6 (*DEFA6*), osteoprotegerin (*OPG*)). Among the other downregulated genes, four encode neurotrophic factors (*Contactin1*, *Ephrin-A5*, Chemokine-Like Receptor 1 (*CMKLR1*) and Neuropeptide Y (*NPY*)), two are involved in signal transduction (G Protein-Coupled Receptor 1 (*GPR-1*), Protein-Tyrosine Phosphatase Nonreceptor-Type 3 (*PTPN3*)), and one, *Cyclin A1*, is required for entry in to the S phase of the cell cycle. A downregulation of the Vascular Endothelial Growth Factor Receptor-3 (*VEGF-R3*) was also observed.

With regard to upregulated genes, again, most of them are involved in the regulation of the immune and monocyte-macrophage systems (IL-17 R, Complement Component Receptor 1 (*CR1*), Tumour Necrosis Factor Receptor 2 (*TNFRSF1B*), *GM-CSF Ralpha*, *MIP-1beta* (Macrophage Inflammatory Protein 1-beta), *CCR-9* chemokines and two factors related to the Th2 function

Table 1
The alteration of gene expression by IdB 1016 treatment

Genes	Fold change (mean±S.D.)
TLR1	0.26±0.16
IL-12 p35	0.26±0.10
GM-CSF	0.26±0.24
TNFRSF11B/OPG	0.34±0.21
NPY	0.35±0.18
Cyclin A1	0.39±0.19
PTPN3	0.42±0.13
DEFA6	0.42±0.15
Contactin 1	0.43±0.13
VEGF-R3	0.44±0.14
Ephrin-A5	0.47±0.10
MIP-1alpha	0.48±0.10
CMKLR1/ChemR23	0.49±0.14
IL-11	0.50±0.13
GPR-1	0.50±0.04
Cyclin G2	2.10±0.70
IL-22	2.29±0.66
DOK1	2.53±0.70
BMP-8	2.66±0.44
Ephrin-A7	2.80±0.25
CCR-9	2.84±0.48
TNFRSF1B	2.96±0.53
IL-10 Ralpha	2.99±0.46
Angiopoieitin-2	3.24±0.65
CR1	3.38±0.06
IL-17 R	3.39±0.45
Cyclo-oxygenase-2	3.56±0.79
GM-CSF Ralpha	3.77±0.99
PRKCB1	4.34±0.83
FGF-16	5.06±0.21
MIP-1beta	5.12±0.13
GAB1	6.05±0.06

S.D., standard deviation.

(*IL-10 Ralpha* and *IL-22*)). Three genes involved in the signal transduction were found overexpressed: two possess a docking function, by amplifying the signal of tyrosine kinases and other signal transducers (GRB2-Associated Binding Protein 1 (*GAB1*) and Docking Protein 1 (*DOK1*)), while Protein Kinase C Beta-1 (*PRKCB1*) is involved in the signalling of growth factors and cytokines, including VEGF [18]. Other upregulated genes included: *cyclo-oxygenase-2*, *Cyclin G2* (involved in slowing of the cell cycle), *Ephrin-A7* (related to the nervous system) and Bone Morphogenetic Protein 8 (*BMP-8*) (involved in bone metabolism). Upregulation of Angiopoietin-2 was also noted; this protein is a naturally occurring antagonist of angiopoietin-1 that competes for binding to the Tie-2 receptor and blocks angiopoietin-1-induced Tie-2 autophosphorylation during vasculogenesis [19]. Finally, Fibroblast Growth Factor 16 (*FGF-16*) was also found to be upregulated; although the FGF family is a potent inducer of the angiogenic process, FGF-16 lacks a signal domain and seems to act as a differentiating agent rather than as a modulator of the angiogenic process [20].

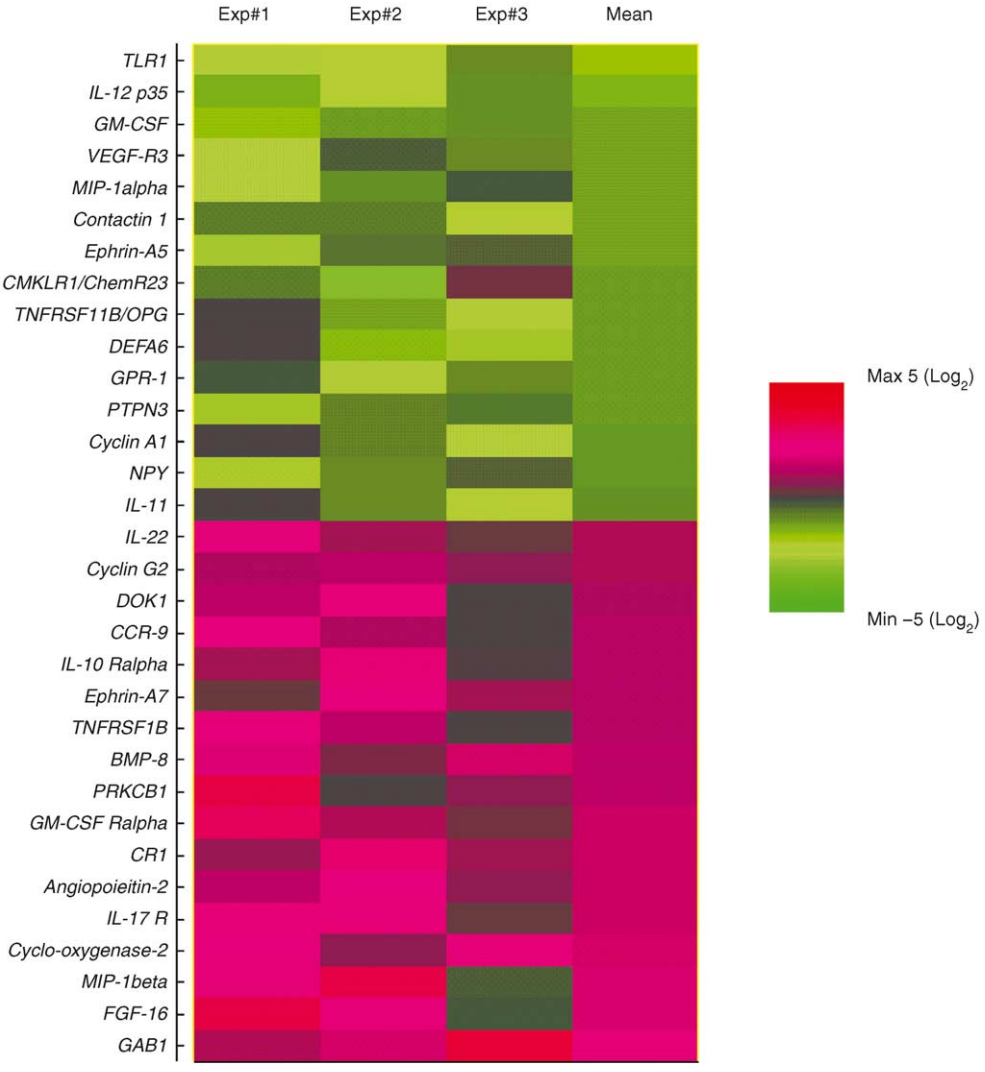

Fig. 2. Image plot of selected genes showing modification in mRNA expression upon IdB 1016 treatment. Three biologically independent experiments were performed and results are shown for each experiment and for the mean of the three. Data are expressed as the ratio of the base 2 log between IdB 1016 treatment over controls. Values higher and lower than 1 indicate up- and downregulated genes, respectively, with a cut-off value of a 2-fold up- or downregulation. Red and green increases indicate the intensity of upregulation and downregulation, respectively. Abbreviations were as follows: Toll-like receptor 1 (*TLR1*), Granulocyte-Macrophage Colony-Stimulating Factor (*GM-CSF*), Vascular Endothelial Growth Factor Receptor-3 (*VEGF-R3*), Macrophage Inflammatory Protein 1-alpha (*MIP-1alpha*), Defensin A6 (*DEFA6*), Chemokine-Like Receptor 1 (*CMKLR1*), Osteoprotegerin (*OPG*), G Protein-Coupled Receptor 1 (*GPR-1*), Protein-Tyrosine Phosphatase, Nonreceptor-Type 3 (*PTPN3*), Neuropeptide Y (*NPY*), Docking Protein 1 (*DOK1*), Tumour Necrosis Factor Receptor 2 (*TNFRSF1B*), Bone Morphogenetic Protein 8 (*BMP-8*), Protein Kinase C Beta-1 (*PRKCB1*), Complement Component Receptor 1 (*CR1*), Macrophage Inflammatory Protein 1-beta (*MIP-1beta*) and GRB2-Associated Binding Protein 1 (*GAB1*).

4. Discussion

We demonstrated in a previous study that IdB 1016 was effective in increasing the CDDP antitumour activity against human ovarian cancer xenografted into athymic mice; however, no significant effects could be observed when the product was administered as monotherapy using a classical dosing schedule for cytotoxic drugs (i.e. q3/4d×4) [11].

In the present study, we extend our previous findings showing that, given with a prolonged schedule treatment as a single agent, IdB 1016 was able to significantly inhibit the growth of human ovarian cancer xenografts. Importantly, the inhibition of tumour growth occurred at clinically achievable plasma levels of silybin. Previous pharmacokinetics studies showed, in fact, that repeated administration to healthy volunteers of doses of up to 2.8 g/day of IdB 1016 produced levels of approximately 0.5 μg/ml (Indena internal report), a value in the range of levels found active in the current study (range 0.2–13.4 μg/ml). This observation is of great importance when considering that, despite the large research effort on the antitumour properties of flavonoids, none of these agents have been introduced in the treatment of cancer, the major problem being their very low bioavailability.

Noteworthy, besides the antitumour activity, a highly favourable toxicity profile was observed in *in vivo* studies. Actually, IdB 1016 has been shown to be very well tolerated and largely free of any adverse effects, both in preclinical and clinical studies [21]. Oral acute toxicity has been demonstrated to be over 5000 mg/kg in rats and monkeys. In repeated toxicity studies (26 weeks of treatment), IdB 1016 proved to be safe in the same animal species up to 1000 mg/kg/day and it did not show any adverse effects when evaluated in reproduction toxicity studies in rats and rabbits. Finally, the good tolerability of the product was confirmed in clinical trials following repeated administration of dosages up to 2.8 g/day to healthy volunteers [21].

Since our previous study [11] suggested the inhibition of angiogenesis as a possible mechanism of action of IdB 1016, determination of VEGF levels and an array analysis were performed in this study in order to get an insight into the role played by the compound in the arrest of vascular growth. However, in our experimental conditions, no modulation of human VEGF production/secretion by IdB 1016 was found, either in tumours from systemically treated mice or *in vitro,* where no differences were determined in conditioned medium from vehicle- and silybin-treated A2780 cells. These findings were confirmed by data obtained from an array analysis of control- and IdB 1016-treated xenografts, that showed no differences between the two groups in the gene expression of VEGF. Taken together, all of these findings indicate that VEGF expression/secretion was not affected in our system and thus this process is not involved in the anti-angiogenic activity of IdB1016. The discrepancy between our data and those reported by Jiang and colleagues [13] on the ability of silymarin to inhibit VEGF secretion by prostate and breast cancer cells, are likely due to the use of a silymarin extract instead of silybin, and also due to other experimental differences, such as the cell line and the duration of drug exposure. We also assessed the concentration of mouse VEGF in the serum of tumour-bearing mice in order to estimate the possible effect of IdB 1016 on host-derived VEGF production; the early vascularisation of the tumour xenograft is in fact, at least partially, due to the initial acute inflammatory reaction and represents an attempt by the host to heal the wound of injection [22]. Actually, like human VEGF production, mouse VEGF production was not different in the control and treated mice.

At least two hypotheses may be drawn from results of the array analysis. A downregulation of *Flt4* (VEGF receptor 3) expression was noticed in ovarian cancer xenografts from IdB 1016-treated mice; interestingly, even if this receptor is mainly involved with its ligands, VEGF-C and VEGF-D, in lymphoangiogenesis [23], it has been found to be overexpressed in invasive breast cancer and to be associated with angiogenesis [24]. In addition, a remarkable upregulation of angiopoietin-2 was noticed upon IdB 1016 treatment. It has been shown that, angiopoietin-1 (Ang1) and 2 (Ang2), their tyrosine kinase receptor, Tie-2, and vascular endothelial growth factors (VEGFs) are critical components of the vasculatory machinery, playing complementary and coordinated roles in the processes of growth and remodelling of normal as well as tumour vessels [25–27]. In several experimental models, Ang2 promotes angiogenesis but, in order to produce such an effect, the increase of Ang2 requires a simultaneous increase in VEGF [27,28]. In the absence of VEGF or following alteration of its signalling, it is likely that overexpression of Ang2 inhibits the angiogenic process by preventing tumour vessel differentiation and maturation. Although both these hypotheses require confirmation at the protein level, we could speculate that, in our experimental model, an increase in Ang2, without a concomitant rise in VEGF levels and/or in the presence of an alteration in its signalling, may, at least partly, explain the anti-angiogenic effect of IdB 1016. Further studies are now ongoing in our laboratory in order to explore the role played by silibyn/IdB 1016.

The modulation of several genes involved in the immune and monocyte-macrophage systems also requires further investigation. Other reports suggest that silybin acts as an immunomodulator [29,30], but such an effect cannot be evaluated in athymic mice and requires experimental work in immunocompetent animals to establish if it could hamper or enhance the IdB 1016 antitumour activity.

In conclusion, taken together, our findings suggest IdB 1016 is a good candidate, with a relevant clinical potential, for use in the management of recurrent ovarian cancer. Besides the antitumour activity observed in *in vivo* relevant animal models, one of the major advantages of the compound in this clinical setting is its favourable toxicity profile, which has a minimal impact on the individual's otherwise good quality of life. A phase II, non-randomised clinical study is now ongoing in our Institute aimed at evaluating the efficacy of daily administrations of 2.8 g IdB 1016 in patients with recurrent ovarian cancer. The dose-selection for the clinical trial was based on the evaluation of plasma drug concentrations associated with target inhibition and antitumour activity in animal studies (see above), this being the recommended approach for such a target-based antiproliferative agent [31].

References

1. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin* 2002, **52**, 23–47.
2. Markman M, Bookman MA. Second-line treatment of ovarian cancer. *the Oncologist* 2000, **5**, 26–35.
3. Morazzoni P, Magistretti MJ, Giachetti C, Zanolo G. Comparative bioavailability of silipide, a new flavanolignan complex in rats. *Eur J Drug Metab Pharmokinet* 1992, **17**, 39–44.
4. Barzaghi N, Crema F, Gatti G, Pifferi G, Perucca E. Pharmacokinetic studies on IdB 1016, a silybin-phosphatidylcholine complex, in healthy human subjects. *Eur J Drug Metab Pharmokinet* 1990, **15**, 333–338.
5. Scambia G, De Vincenzo R, Ranelletti FO, *et al*. Antiproliferative effect of silybin on gynaecological malignancies: synergism with cisplatin and doxorubicin. *Eur J Cancer* 1996, **32A**, 877–882.
6. Bhatia N, Zhao J, Wolf DM, Agarwal R. Inhibition of human carcinoma cell growth and DNA synthesis by silybinin, an active constituent of milk thistle: comparison with silymarin. *Cancer Lett* 1999, **147**, 77–84.
7. Zi X, Zhang J, Agarwal R, Pollak M. Silibinin up-regulates insulin-like growth factor-binding protein 3 expression and inhibits proliferation of androgen-independent prostate cancer cells. *Cancer Res* 2000, **60**, 5617–5620.
8. Singh RP, Dhanalakshmi S, Tyagi AK, Chan DC, Agarwal C, Agarwal R. Dietary feeding of silibinin inhibits advance human prostate carcinoma growth in athymic nude mice and increases plasma insulin-like growth factor-binding protein-3 levels. *Cancer Res* 2002, **62**, 3063–3069.
9. Tyagi A, Agarwal C, Agarwal R. The cancer preventive flavonoid silibinin causes hypophosphorylation of Rb/p107 and Rb2/p130 via modulation of cell cycle regulators in human prostate carcinoma DU145 cells. *Cell Cycle* 2002, **1**, 137–142.
10. Tyagi A, Bhatia N, Condon MS, Bosland MC, Agarwal C, Agarwal R. Antiproliferative and apoptotic effects of silibinin in rat prostate cancer cells. *Prostate* 2002, **53**, 211–217.
11. Giacomelli S, Gallo D, Apollonio P, *et al*. Silybin and its bioavailable phospholipid complex (IdB 1016) potentiate in vitro and in vivo the activity of cisplatin. *Life Sci* 2002, **70**, 1447–1459.
12. Tyagi AK, Singh RP, Agarwal C, Chan DC, Agarwal R. Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth Inhibition, G2-M arrest, and apoptosis. *Clin Cancer Res* 2002, **8**, 3512–3519.
13. Jiang C, Agarwal R, Lü J. Anti-angiogenic potential of a cancer chemopreventive flavonoid antioxidant, Silymarin: inhibition of key attributes of vascular endothelial cells and angiogenic cytokine secretion by cancer epithelial cells. *Biochem Biophys Res Commun* 2000, **276**, 371–378.
14. Corbett T, Valeriote F, LoRusso P, *et al*. In vivo methods for screening and preclinical testing. In Teicher BA, ed. *Anticancer Drug Development Guide*. Totowa, NJ, Humana Press, 1997, 75–99.
15. Obermair A, Kucera E, Myerhofer K, *et al*. Vascular endothelial growth factor (VEGF) in human breast cancer: correlation with disease-free survival. *Int J Cancer* 1997, **74**, 455–458.
16. Martinelli EM, Morazzoni P, Livio S, Uberti E. Liquid chromatographic assay of silybin in human plasma and urine. *J Liquid Chromatogr* 1991, **14**, 1285–1296.
17. Eisen MB, Brown PO. DNA arrays for analysis of gene expression. *Methods Enzymol* 1999, **303**, 179–205.
18. Teicher BA, Alvarez E, Menon K, *et al*. Antiangiogenic effects of a protein kinase Cbeta-selective small molecule. *Cancer Chemother Pharmacol* 2002, **49**, 69–77.
19. Kim I, Kim JH, Ryu YS, Jung SH, Nah JJ, Koh GY. Characterization and expression of a novel alternatively spliced human angiopoietin-2. *J Biol Chem* 2000, **275**, 18550–18556.
20. Miyake A, Konishi M, Martin FH, *et al*. Structure and expression of a novel member, FGF-16, on the fibroblast growth factor family. *Biochem Biophys Res Commun* 1998, **243**, 148–152.
21. Malandrino S, Pifferi G. IdB 1016 Silybin Phosphatidylcholine Complex. *Drugs Future* 1990, **15**, 226–227.
22. McLeskey SW, Tobias CA, Vezza RV, Filie AC, Kern FG, Hanfelt J. Tumor growth of FGF or VEGF transfected MCF-7 breast carcinoma cells correlates with density of specific microvessels independent of the transfected angiogenic factor. *Am J Pathol* 1998, **153**, 1993–2006.
23. Li X, Eriksson U. Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. *Int J Biochem Cell Biol* 2001, **33**, 421–426.
24. Valtola R, Salven P, Heikkila P, *et al*. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 1999, **154**, 1381–1390.
25. Hanahan D. Signaling vascular morphogenesis and maintenance. *Science* 1997, **227**, 48–50.
26. Maisonpierre PC, Suri C, Jones PF, *et al*. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 1997, **277**, 55–60.
27. Holash J, Maisonpierre PC, Compton D, *et al*. Vessel Cooption, regression, and growth in tumours mediated by angiopoietins and VEGF. *Science* 1999, **284**, 1994–1998.
28. Kämpfer H, Pfeilschifter J, Frank S. Expressional regulation of angiopoietin-1 and-2 and Tie-1 and-2 receptor tyrosine kinases during cutaneous wound healing: a comparative study of normal and impaired repair. *Lab Invest* 2001, **81**, 361–373.
29. Meroni PL, Barcellini W, Borghi MO, *et al*. Silybin inhibition of human T-lymphocyte activation. *Int J Tissue React* 1988, **10**, 177–181.
30. Katiyar SK. Treatment of silymarin, a plant flavonoid, prevents ultraviolet light-induced immune suppression and oxidative stress in mouse skin. *Int J Oncol* 2002, **21**, 1213–1222.
31. Rowinsky EK. The pursuit of optimal outcomes in cancer therapy in a new age of rationally designed target-based anticancer agents. *Drugs* 2000, **60**(Suppl. 1), 1–14.