original article

Phase I study of apoptosis gene modulation with oblimersen within preoperative chemotherapy in patients with primary breast cancer

J. Rom[1]*, G. von Minckwitz[2], F. Marmé[1], B. Ataseven[3], D. Kozian[4], M. Sievert[4], B. Schlehe[1], F. Schuetz[1], A. Scharf[1], M. Kaufmann[2], C. Sohn[1] & A. Schneeweiss[1]

[1]Department of Gynecology and Obstetrics, University of Heidelberg, Heidelberg; [2]Department of Gynecology and Obstetrics, University of Frankfurt, Frankfurt; [3]Departement of Gynecology and Obstetrics, Red Cross Womens Hospital, Munich and [4]Medical Oncology, Sanofi-Aventis GmbH, Berlin, Germany

Expression of the Bcl-2 protein confers resistance to chemotherapy-mediated apoptotic signals in patients with breast cancer. We investigated effects of Bcl-2 down-regulation by the Bcl-2 antisense oligodeoxynucleotide oblimersen in breast tumor biopsies. Oblimersen targets Bcl-2 messenger RNA (mRNA), down-regulates Bcl-2 protein translation and enhances antitumor effects of subtherapeutic chemotherapy doses. Within a phase I trial, we administered escalating doses of oblimersen (3, 5 or 7 mg/kg/day) as continuous infusion on days 1–7 in combination with standard-dose docetaxel (Taxotere), Adriamycin and cyclophosphamide (TAC) on day 5 as preoperative chemotherapy in 28 patients with T2–4 tumors. Effects of oblimersen were evaluated in tumor biopsies and peripheral blood mononuclear cells (PBMCs) 4 days after start of oblimersen and before TAC treatment by quantitative microfluidic real-time PCR. Read-outs consisted in measurement of Bcl-2 mRNA modulations and of 18 putative predictive markers. Two of 13 patients showed a diminution of Bcl-2 transcripts after 4 days of treatment with oblimersen 5 mg/kg/day. PBMCs could not be evaluated as a surrogate tissue because no qualified RNA could be isolated. Nevertheless, we demonstrated feasibility to process clinical samples and to obtain good quality RNA from tumor biopsies and indicated the potential of oblimersen to lower Bcl-2 mRNA in breast cancer.
Key words: apoptosis, breast cancer, oblimersen

introduction

Chemotherapy and surgery are two main pillars of breast cancer therapy, but relative timing has been intensively investigated. Preoperative or neoadjuvant chemotherapy, which can be regarded as an *in vivo* chemosensitivity test [1], confers some advantages. First, reduction of tumor size can improve the rate of breast-conserving surgery and cosmetic results. Secondly, it was hypothesized that earlier use of cytotoxic drugs might reduce the number of chemoresistant cell clones. Knowing that development of chemoresistance is a major obstacle in the therapeutic intervention of most cancers, preoperative chemotherapy could be advantageous. Thirdly, a rapid and direct assessment of response or resistance to a particular treatment regimen can accelerate evaluation and validation of new predictive factors, which will allow the development of tailored treatment strategies.

One possible mechanism of drug resistance is related to the failure of cells to engage apoptosis, i.e. programmed cell death, associated with many cytotoxic agents. Specifically, several members of the Bcl-2 family of proto-oncogenes (in particular Bcl-2 itself) have been described as antiapoptotic proteins and have been shown to be key regulators of the intrinsic cell death pathway. Bcl-2 protects cells from a variety of external stresses, including chemotherapy and radiation [2–4], and its expression has been correlated with poor prognosis in a number of malignancies [5, 6]. Moreover, Bcl-2 is expressed in the majority of breast cancers. For these reasons, Bcl-2 became an attractive target for molecular targeting approaches. An inhibitor leading to a decrease of this protein could act as a chemosensitizer of tumor cells to apoptotic stimuli. Oblimersen is a phosphorothioate 18-mer antisense oligonucleotide (5′-TCTCCCAGCGTGCGCCAT-3′) selectively directed at the Bcl-2 open reading frame. It was designed to specifically bind to the Bcl-2 messenger RNA (mRNA), thus blocking the production of the respective protein, and consequently may enhance the antitumor efficacy of standard cytotoxic chemotherapy [7, 8].

Since it is known that the combination of oblimersen with docetaxel or cyclophosphamide leads to synergistic antitumor effects in solid tumors and breast cancer cell lines [9–13], a phase I study was carried out to investigate the combination of oblimersen with a standard chemotherapy consisting of

Correspondence to: Dr J. Rom, Department of Gynecology and Obstetrics, University of Heidelberg, Vossstrasse 9, D-69115 Heidelberg, Germany. Tel: +49-6221-56-37987; Fax: +49-6221-56-7066; E-mail: joachim.rom@med.uni-heidelberg.de

docetaxel, Adriamycin and cyclophosphamide (TAC) as preoperative treatment in primary breast cancer. Results of the clinical and pharmacokinetic end points of this trial have been published elsewhere [14]. The preoperative setting was chosen to have access to tumor cells before and after *in vivo* treatment, which would enable us to evaluate predictive factors for response and resistance within a companion translational study. The translational study was restricted to genotyping of patients for *Bcl-2* and to transcriptional expression analyses before and after treatment with the antisense but before the administration of TAC. Therefore, this protocol did not allow us to study the combination effect of oblimersen with the preoperative chemotherapy.

- End points of the translational companion protocol were (i) to analyze Bcl-2 expression in breast tumor biopsies at diagnosis and during the course of treatment in order to evaluate Bcl-2 expression as a predictive marker for response, (ii) to evaluate peripheral blood mononuclear cells (PBMCs) as a surrogate tissue because accessibility and quality of breast cancer tissues itself is often limited, (iii) to describe the impact of oblimersen on apoptosis mechanisms in breast cancer cells in order to identify molecular markers for the prediction of treatment outcome, (iv) to evaluate the role of polymorphisms and gene amplification of selected candidate genes sensitive to oblimersen treatment.

patients, materials and methods

treatment plan

The study plan was built to evaluate the recommended dose of oblimersen as preoperative systemic therapy using three cohorts of nine patients with primary advanced breast cancer (stage T2–4, N0–3, M0). The dose of the investigational agent was escalated until the occurrence of dose-limiting toxic effects. All patients received one cycle of oblimersen at a dose of 3, 5 or 7 mg/kg on days 1–7 as continuous i.v. infusion with a fixed dose of docetaxel (Taxotere, Sanofi-Aventis GmbH, Germany) 75 mg/m^2 on day 5, Adriamycin 50 mg/m^2 on day 5 and cyclophosphamide 500 mg/m^2 on day 5 (OTAC), followed by five cycles of standard TAC without oblimersen on day 1 every 3 weeks before surgery [14].

blood serum samples and tissue collection

Fresh frozen tissue samples from patients were collected at diagnosis and at day 4 of the first cycle before starting TAC treatment for biomarker/target gene studies. The tissue collection was carried out according to the following protocol: four to six core biopsies of the primary cancers from every patient were taken at the hospital using standard procedure. Samples were taken after patients had received local anesthesia with the same entry point, but reorienting the needle (one biopsy = 20 mg tissue). One to two core biopsy specimens were fixed for diagnostic analyses and remained at the hospital. Two biopsies were immediately snap frozen in liquid nitrogen, stored at −80°C at the hospital.

sample preparation and analysis

PBMCs were prepared at the hospitals from whole blood, at diagnosis and at day 4 of first cycle. Briefly, 16 ml of lymphocyte separation medium (LSM) was pipetted into two 50-ml centrifuge tubes (Leuco Sep, Greiner, Germany) and then centrifuged for 30 min at 1000 *g*. The heparinized blood was diluted 1 : 1 in phosphate-buffered saline (PBS) and carefully

layered over the LSM. The tubes were centrifuged for 30 min at 800 *g*. The upper phases (phases above barrier in the tube) were transferred into new containers and filled 1 : 1 with PBS and again centrifuged for 6 min at 500 *g*. After centrifugation, supernatants were discarded and both cell pellets were resuspended with 1–5 ml PBS and brought together in a new 50 ml container. PBS was added up to 50 ml. After centrifugation, the cell pellets were again resuspended with 3 ml PBS and 1 ml was given in a new probe vial (three lockable vials required). Vials were centrifuged for 1 min at 12 000 *g*, supernatant was discarded and vials were snap frozen and stored at −80°C. As indicated within the approved 'Commission Nationale de l'Informatique et des Libertes' (CNIL) dossier, all samples were barcoded and registered in SPECTRUM software.

Biopsies and PBMCs were lysed in Qiagen buffer and centrifuged and supernatants were taken. This step was done twice for tumor biopsies to maximize final RNA yield. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), including a DNAse I treatment step [27 Kunitz units per column during 30 min at room temperature; RNAse-Free DNAse Set (Qiagen)] and resuspended in RNAse-free treated water. RNA was quantified by absorbance at 260 nm (NanoDrop ND1000); then, its integrity was assessed by microchip capillary electrophoresis on Agilent 2100 bioanalyzer.

Only 250 ng of total RNA was used to generate single-stranded complementary DNA (cDNA) in 50 μl of reaction volume (TaqMan Reverse Transcription Reagents; Applied Biosystems Inc., Foster City, CA) using oligo dT and random hexamer primers at final concentration of 2.5 μM. The cycling parameters consisted of the following: 25°C for 10 min, 42°C for 50 min and 95°C for 2 min. Reactions without reverse transcriptase were systematically carried out in parallel. Quality of cDNA synthesis reaction and absence of contaminating genomic DNA were assessed by amplifying β2-microglobulin and cyclophilin A sequences by TaqMan real-time quantitative PCR (QPCR). Primers are listed in Table 1. As a negative control, samples lacking reverse transcriptase (RT) during cDNA synthesis were used. Up to 10 copies of genomic DNA is accepted which correspond to following criteria:

cDNA quality control (QC) criteria:
β2-microglobulin: +RT cDNA Ct < 24 −RT cDNA Ct > 31
Cyclophilin A: +RT cDNA Ct < 24 −RT cDNA Ct > 29
(Ct, number of cycles necessary to detect an amplification signal)

Each Taqman Gene Expression Assay consists of a forward and reverse primer at a final concentration of 900 nM and a Taqman probe labeled with fluorogenic dyes fluoresceine and rhodamine (attached at the 5′ and 3′ ends of the probe, respectively; Applied Biosystems), at 250 nM final concentration. The assays are mentioned to be gene specific and have been designed to span exon–exon junction. Samples were run using the 7900HT system with a Taqman Low Density array upgrade, according to the Manufacturer's instructions. In short, 2 μl of single-stranded cDNA (to final concentration of 100 ng starting RNA) was combined to 48 μl water and 50 μl Taqman Universal PCR Master Mix, followed by loading of 100 μl sample per port (each well contains 2 ng starting RNA converting into cDNA). Thermal cycling conditions were as follows: 50°C for 2 min, 94.5°C for 10 min, 97°C for 30 s and 59.7°C for 1 min, for a total of 40 cycles. The Ct was automatically determined by the SDS 2.1 software package (Applied Biosystems). Results files were loaded in a study and results from each card were quantified separately using automatic baseline and threshold. Gene expression values were calculated based on the δδCt method, resulting in a relative expression level: relative abundance of genes = $2^{(-\Delta Ct)}$, where ΔCt is the difference between the Ct of target and the arithmetic mean of Cts of reference genes. Genes were considered as significantly expressed and their transcript measurable if their corresponding Ct value ≤35 and delta Ct between duplicates was up to 1.

Table 1. Primer/probe sets used for sample quality control

Design ID	Set No.	Reagent ID	Reagent type	Sequence	Length
1284	1	hAVE000B2MTP02.1	P	TATCTGAGCAGGTTGCTC (MGB)	18
1284	1	hAVE000B2MTF02.1	F	TTGAGTGCTGTCTCCATGTTTGAT	24
1284	1	hAVE000B2MTR02.1	R	CTAAGTTGCCAGCCCTCCTAGAG	23
		Cyclophilin A	P	TCTCGCTGCAGTTCCCTTTGGGTTC	25
		Cyclophilin A	F	TGCTCGCAGTATCCTAGAATCTTTG	25
		Cyclophilin A	R	AGGCATGGGAGGGAACAAG	19
589	1	hAVE00APM1TP01.1	P	ACCTTCAGCCCCGGGTACTCCG	22
589	1	hAVE00APM1TF01.1	F	CCTAAGGGAGACATCGGTGAAAC	23
589	1	hAVE00APM1TR01.1	R	CCTTTCCTGCCTTGGATTCC	20

F and R represent forward (upstream) and reverse (downstream) primers, respectively. P represents the probe with fluorogenic dyes fluorescine and rhodamine (or MGB) attached at the 5′ and 3′ ends of the probe, respectively. For each primer set, references in the proliferation path and targeting system database are indicated in the three left-hand columns, when appropriate. MGB, minor groove binder.

Modulation of expression was analyzed with the following criteria: minimal fold change considered is 1.8 (corresponding to 1.8 for an up-regulation and 0.56 for a down-regulation). The Bioanalysis annotation process was run to identify and integrate all known sequences related to each gene of interest. Basic (BLAST, Sim4, Swat, ClustalW and InterProScan) and sophisticated bioinformatics tools—developed by the Vitry Genomic Sciences bioanalysis team—have been used to analyze the sequences and data around the genes of interest. The following databases were screened for the annotation: Mouse and Human GenBank DNA/RNA/HTC, Mouse/Human RefSeq Genomic, Mouse/Human RefSeq RNA, RepBase, Mouse and Human Derwent Genseqn and Mouse and Human GenBank patent. All the bioanalysis steps were carried out semiautomatically and then subjected to manual expert analysis to guarantee high quality and reliability of data at Sanofi-Aventis Laboratories in Paris, France.

selection of genes

A subset of genes involved in balancing apoptotic and antiapoptotic signal was proposed to investigate effect of oblimersen (e.g. Bcl-2, Bcl-xl, Bcl-w, Bak, Bax, Bad, BIRC 2/3/4 and DIABLO). In addition to the markers selected for this study, three genes (β2-microglobulin, ribosomal protein L37a and ribosomal RNA 18S) were plotted as endogenous controls and were used as a reference to calculate expression level of target genes. For all the genes and the endogenous controls, predesigned Taqman probe and primer sets were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. Once selected, the sets were ordered and factory loaded into 384 wells of Taqman Low Density Array by the provider. EMSY oncogene, which is a modulator of BRCA2 expression [15], was added as it was recently demonstrated that it is frequently amplified in breast cancer. Because RNAse H is claimed to be a key component in the mechanism of inhibition of gene expression by antisense phosphorothioate oligonucleotides, RNAse H1 and RNAse H2 were included in the list of markers tested [16].

results

Twenty-eight patients were enrolled into this phase I study from June 2004 to September 2005 (University of Heidelberg 20 patients, University of Munich five patients and University of Frankfurt three patients). Nine patients received oblimersen 3 mg/kg/day (cohort I), nine patients 5 mg/kg/day (cohort II) and 10 patients 7 mg/kg/day (cohort III). Median age was 50 years (range 35–64), median Karnofsky performance status was 90% (range 90–100), 61% of patients had clinically T2 tumors, 68% had a ductal-invasive histology and 86% were node positive. Overall, 43% of tumors were poorly differentiated, 36% hormone receptor negative and 18% overexpressed Her2. Details of patient and tumor characteristics are given in Table 2.

bioanalysis and selection of reagents

The screening of the human DNA and transcript databases allowed the determination of all the known mRNAs related to the 19 genes of interest. Globally, several isoforms are reported for 11 of these genes. Because the catalog contains more than one AOD per gene, it is necessary to select the one that will track all important splice variants.

Based on the information provided by the inventory of databases, the catalog of available probe and primer sets was screened. In most cases, the objective was to identify a set of reagents that would permit to track all the isoforms of each gene, what we call further a 'common set'. For 14 of these genes, a unique common set was available from the provider (except for mcl-1) and was thus selected. For bcl-x, two isoforms are known: bcl-xs and bcl-xl. Among the two sets available, one should be specific to bcl-xL and the other to bcl-xS. We have checked this assumption experimentally. Their profiles on a panel of 63 human tissues were compared with the profiles of two homemade specific designs considered as reference (data not shown). Because each AOD profile was identical to corresponding in-house design, we confirmed that each AOD is specific to one of the splice variant.

For the four remaining genes (Bcl-2, Bax, Bim and Htr-A2), a refined analysis was carried out to choose the sets that should track all the splice variants. Using information provided in the catalog and the graphic compilation resulting from bioanalysis, we localized the AODs along the sequences and identified the ones that should track all the splice variants of a given gene. For Bim, only one of the three sets was common to all the isoforms. For Bax, we selected two representative sets. For Htr2A, one set of two was selected arbitrary, as they were both common designs. For Bcl-2, three sets were available, whereas two isoforms (called A and B) are described. Based on the information registered in the catalog, one of these AODs should

Table 2. Patient and tumor characteristics

Characteristics	Dose level			
	3 mg/kg/day, $n = 9$	5 mg/kg/day, $n = 9$	7 mg/kg/day, $n = 10$	Total (%), $n = 28$
Age, years				
Median	47	49	55.5	50
Range	38–54	35–61	41–64	35–64
Karnofsky performance status, %				
Median	90	90	90	90
Range	90–100	90–100	90–100	90–100
Tumor size				
T2	6	4	7	17 (61)
T3	2	5	2	9 (32)
T4	1	0	1	2 (7)
Nodal status				
N0	2	1	1	4 (14)
N1	6	7	8	21 (75)
N2	0	1	1	2 (7)
N3	1	0	0	1 (4)
Histological grading				
Well differentiated	1	0	1	2 (7)
Moderately differentiated	4	6	4	14 (50)
Poorly differentiated	4	3	5	12 (43)
Hormone receptor status				
ER and/or PgR positive	7	6	5	18 (64)
ER and PgR negative	2	3	5	10 (36)
Her2 status				
Score 3+ or 2+/FISH positive	2	2	1	5 (18)
Score 0, 1+ or 2+/FISH negative	7	7	9	23 (82)
Response rates[a]				
Clinical CR	3	2	2	7 (28)
Clinical PR	3	3	1	7 (28)
Clinical NC	1	1	3	5 (20)
Pathological CR	3	2	0	5 (20)
Pathological PR	4	4	6	14 (56)
Pathological NC	0	2	1	3 (12)

[a]Not all data available.
ER, estrogen receptor; PgR, progesterone receptor; CR, complete remission; PR, partial response; NC, no change.

be a common design, whereas the two others should be specific to each of the splice variants A and B. Because the location of probe and primer sets was not accurate enough, we decided to check for the specificity of these sets. As for bcl-x mentioned above, we have designed some Taqman reagents for each of the isoforms. Both these homemade sets and the three AODs were profiled on a panel of 63 human tissues. First, the AOD corresponding to a common design did not provide relevant data and thus was not validated. Secondly, the in-house sets targeting isoforms A and B demonstrated some tissue specificities. In particular, the isoform A is much more expressed in thyroid gland and fetal aorta than the isoform B. Conversely, the isoform B is much more expressed in bone marrow than the isoform A. Based on these tissue

particularities, we were able to discriminate both isoforms and to conclude that the two AODs are specific to isoform A. Final selection was done on the efficacy criterion estimated on a standard curve: PCR presents 98% of efficacy for the HS00153350, whereas Hs00608023 demonstrated only 90% of efficacy. In conclusion, we have selected 24 probe and primer sets, which will allow us to measure the level of expression of 19 genes and three potential genes of reference.

collection of samples

After completion of the recruitment phases, 86 samples were collected at the hospitals following standard procedures: 44 PBMC pellets and 42 tissue biopsies. Regarding the three dose groups of oblimersen with 3, 5 and 7 mg/kg/day, biopsies from six, eight and eight patients, respectively, had been collected. In 13 patients, cDNA was available from the two different time points. All the samples were registered and barcoded into SPECTRUM database, according to the CNIL process requirements.

RNA isolation, reverse transcription and QCs

Isolation of RNA was carried out with these 86 samples. RNA QC was carried out regarding both quality and quantity aspects. None of the RNAs obtained from PBMCs were qualified, as they are in an advanced degradation state. Conversely, RNA isolated from tumor biopsies was of good quality for Taqman profiling. Only 8 of 42 were degraded. In addition, we observed a lower RNA yield than those obtained from human breast tumors from other sources: globally, we obtained 0.5 µg/mg of tumor biopsies, whereas we expected ~1 µg/mg of tissue. Finally, 34 samples were successfully reverse transcribed for transcript profiling. However, this means that we get evaluable QCed RNA at the two time points (diagnosis and day 4) of only 13 patients. For eight patients, we only have part of samples (for two of them, one point of time only was collected and for the six others, RNA did not pass QC) and for one patient, both biopsies failed at the RNA QC step.

Because the protocol of the study was restricted following arrest of collaboration with Genta, histological control of tissues was only carried out in the hospitals. The corresponding histopathological reports are stored in Vitry, France. However, the most critical point in breast biopsies is the risk of contamination by adipose tissue. To document this possibility, and as part of QC, we have determined the level of expression of adiponectin (APM1) in our samples. This transcript was previously identified as a potential biomarker specific to adipose tissue, compared with different breast and/or mammary gland samples. Despite the heterogeneity of OTAC samples [median = 4.3; standard deviation (SD) = 33], we showed that expression of APM1 is equivalent to currently used breast samples (median = 8; SD = 60), normal or tumoral, and is clearly smaller than expression detected in a pool of adipose tissue. In conclusion, we estimate that all OTAC samples are not dramatically contaminated by adipose tissue.

quantitative expression analysis of transcripts

The 34 samples qualified previously were characterized using a Taqman microfluidic card. After verifying that required

RNAse H is present, the first level of analysis is to check whether Bcl-2 transcripts are silenced by the oblimersen Bcl-2 antisense. The first observation is that RNAse H1 and H2 are expressed in all the patients, in particular at the diagnosis point of time. However, levels of expression of RNAse H transcripts are highly variable. Their expressions normalized to RPL37a vary from 3.4 to 23.5 (equivalent Ct: 30–26) and from 2.2 to 69.6 (equivalent Ct: 30.6–25) for RNAse H1 and RNAse H2, respectively. All patients demonstrate expression of Bcl-2. However, the basal level of expression of Bcl-2 transcripts is highly variable among patients. Its expression normalized to RPL37a varies from 1.5 to 80 (equivalent Ct: 30.6–24.6) (Figure 1). Independent of this heterogeneity we were not able to demonstrate an obvious effect of oblimersen treatment. Only 2 of 13 patients show a significant down-regulation of Bcl-2 transcripts after 4 days of oblimersen treatment. However, patients 9 (pair OBL000057 and OBL000058) and 12 (pair OBL000087 and OBL000088) belong to the group treated with 5 mg/kg/day of oblimersen antisense. Seven patients (28%) achieved a complete remission (three patients in cohort I, two patients in cohort II and two patients in cohort III) and also three patients (28%) a partial remission for an overall clinical response rate of 56%. Five (20%) showed a pathological complete remission and 14 (56%) patients a partial remission for an overall pathological response rate of 76% (Table 2).

To go more in depth in the analysis, we have calculated the fold change between the two points of time for each of the 13 patients (Figure 2). For this analysis, the cut-off for the fold change was fixed to 1.8 for overexpression and conversely to 0.56 for underexpression. According to these criteria, we verify that both patients 9 and 12 demonstrate a down-regulation of Bcl-2 transcripts of at least two-fold (Figure 3).

The same calculations were done for the 20 other genes. However, because we did not demonstrate any clear effect of the treatment, the impact on other genes is not likely to be of much significance. We can just notice that patients 12 and 21

show a general diminution of all transcripts (also the reference genes) that indicate a problem in one step of the protocol and/ or manipulation rather than a biological consequence of the treatment.

discussion

Under physiological conditions, a balance exists between pro- and antiapoptotic signals. However, in a cancerous state, a dysregulation of this balance may occur, inhibiting the cells' ability to respond to the apoptotic signal induced by anticancer family members. Expression of the Bcl-2 antiapoptotic protein confers resistance to various apoptotic signals [17, 18] and leads to a reduced response rate to chemotherapy and a shorter time to progression and overall survival [12, 19–21]. Numerous studies have documented the effect of Bcl-2 manipulation using gene or antisense oligodeoxynucleotide (AS ODN) therapy on the susceptibility of tumor cells to apoptotic stimuli [21–24]. Bcl-2 AS ODNs have been shown to induce tumor growth suppression independent of additional chemotherapy in murine and human xenograft solid tumor models [21, 22, 23, 25]. In this context, AS ODN directed against Bcl-2 has been investigated as therapeutic agents, and one such ODN, oblimersen, has progressed to the stage of phase II clinical testing [6, 9, 26]. Stable plasma concentration levels of oblimersen could be achieved after 12–24 h of i.v. infusion [27–29]. The results of the present study show that on clinical biopsies from human breast cancer and after 4 days of treatment with oblimersen infusion at 3, 5 or 7 mg/kg/day, we do not detect any significant Bcl-2 transcriptional down-regulation. Only 2 of 13 (15%) assessable patients present a lower expression of Bcl-2 transcripts, both received oblimersen at a dose of 5 mg/kg/day.

Certain AS ODNs also showed immune stimulatory effects in cancer patients [30]. The uptake of ODNs is higher in malignant cells and cell lines than in primary cells related to
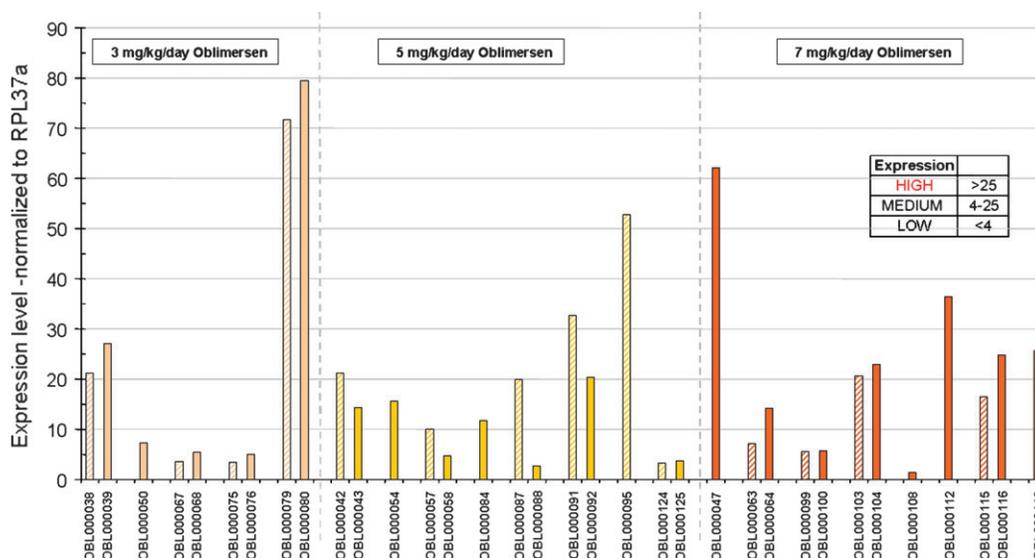
Figure 1. Bcl-2 transcripts level in 21 patients. Bcl-2 expression level was estimated by quantitative real-time PCR on LDA and normalized to RPL37a. Sample pairs, at diagnosis (hatched bars) and 4 days after oblimersen infusion (filled bars), are classified regarding dose group.

Oblimersen dose	Patient	BCL-2-Hs00153350_m1	BCL-xl-Hs00236329_m1	BCL-xl-Hs00169141_m1	BCL-w-Hs00187848_m1	Bim-Hs0078019_s1	BAX-Hs00180269_m1	BAX-Hs0075184_s1	BAD-Hs00188930_m1	BID-Hs00609630_m1	BAK1-Hs00832876_g1	DIABLO-Hs00219876_m1	AKT1-Hs00178289_m1	AKT2-Hs00609846_m1	Htr-2-Hs00376860	RNASEH1-Hs00268000_m1	RNASEH2A-Hs00197370_m1	MCL1-Hs0172036_m1	BIRC2-Hs00357350_m1	BIRC3-Hs00154109_m1	BIRC4-Hs0074522_s1	Bmsy-Hs00220187_m1
3 mg / kg	1	1,3	1,0	1,2	0,9	0,9	0,7	0,8	0,9	0,8	0,9	0,9	0,8	1,0	0,7	0,9	1,8	0,6	0,8	0,8	1,0	1,1
	3	1,5	1,3	1,7	1,0	1,4	1,0	1,1	0,9	0,9	1,0	0,9	1,2	1,2	0,9	1,0	0,7	1,1	1,2	1,8	1,1	0,9
	5	1,5	1,1	0,8	1,3	1,5	0,8	1,2	0,8	1,3	0,6	1,1	1,5	1,2	1,0	1,3	0,7	1,3	1,1	1,4	1,3	1,4
	6	1,1	1,2	1,3	1,2	0,9	1,3	1,3	0,9	1,2	1,0	1,0	1,3	1,0	1,1	1,2	0,9	1,6	1,2	1,4	1,3	1,6
5 mg / kg	7	0,7	0,8	0,9	1,0	0,6	0,5	0,6	0,9	0,7	0,6	0,7	0,5	0,8	0,9	0,8	0,5	0,8	0,8	0,8	0,6	0,9
	9	0,5	1,5	1,8	1,3	0,9	1,3	1,2	2,9	1,1	1,1	1,4	2,0	1,3	0,9	1,5	1,5	1,3	1,2	0,4	1,4	2,3
	12	0,1	0,3	0,6	0,2	0,3	0,4	0,3	0,3	0,2	0,1	0,3	0,3	0,3	0,1	0,2	0,1	0,1	0,4	0,0	0,2	0,4
	13	0,6	1,1	1,5	0,7	0,6	0,8	0,7	1,1	0,8	0,9	1,2	0,9	0,8	0,7	1,0	0,7	0,4	1,1	0,4	1,1	0,5
	10	1,1	1,0	1,1	0,9	0,7	0,8	0,7	1,4	1,0	1,3	0,8	1,0	0,7	1,0	0,6	0,9	0,7	1,3	1,2	0,9	
	16	2,0	1,4	1,4	1,8	1,5	1,1	1,2	1,2	1,2	1,5	1,2	1,5	1,7	1,4	1,4	0,7	1,5	1,7	2,7	1,8	1,2
7 mg / kg	17	1,0	1,9	2,0	1,5	1,5	1,6	1,5	1,9	1,4	1,6	1,3	1,5	1,8	1,5	1,2	2,2	1,7	1,8	0,7	1,9	1,1
	18	1,1	1,3	1,7	1,5	1,5	0,9	1,1	1,2	1,1	1,2	1,3	1,0	0,9	1,2	1,1	1,1	1,5	1,1	0,8	1,3	1,0
	21	1,5	0,4	0,4	0,9	0,7	0,5	0,6	0,3	0,1	0,4	0,4	0,4	0,5	0,4	0,5	0,2	0,4	0,2	0,2	0,7	0,3

Figure 2. Modulation of expression was calculated for the 21 genes. Fold change was calculated between expression at diagnosis and 4 days after oblimersen infusion in 13 patients. Significant modulations estimated according to our criteria are written in red for the activation and in green for the repression.
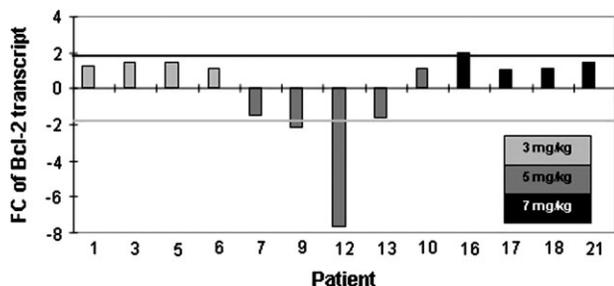

Figure 3. Modulation of expression of Bcl-2 transcripts in 13 patients. The fold change of Bcl-2 expression level was calculated between the diagnosis and 4 days after oblimersen infusion. Patients are classified regarding dose group as indicated by the color code. The cut-off value fixed for this analysis is symbolized by dark gray and light gray lines for up- and down-regulation, respectively.

cellular activation [31]. Mode of action of AS ODNs is based on their ability to bind the complementary sequences in the target mRNA. The resulting heteroduplexes can then be cleaved by RNAse H, leading to disruption of transcripts. We have checked that the absence of effect observed in patients is not due to absence of RNAse H, and in particular of RNAse H2 [16]. In the same way, we have verified that all patients exhibit a basal level of expression of Bcl-2. In all cases, level of expression of these transcripts is compatible with a reliable read-out by QPCR (Taqman), without reaching the limit of the technology. A critical point observed here is the heterogeneity among the patients, considering the level of transcripts of Bcl-2 and RNAse H1 and H2. To reduce this variability, we compared different methods of normalization of the data. Considering a unique gene or a pool of the three genes included in the study (β2-microglobulin, ribosomal protein L37a and ribosomal RNA 18S) leads to the same results. The small number of patients included in this study would not allow us to demonstrate any clear correlation between the level of Bcl-2 and the efficacy of oblimersen. However, the relevance of

analyzing gene expression profiles of treated patients with breast cancer for the identification of responders and nonresponders in a clinical setting has been shown recently [32]. By determining the gene expression level of a selected subset of genes, patients could be classified with 88% accuracy.

Thus, it is important to check the correlation with the clinical outcome and the responding status of each patient and to correlate the responding patients to other genes functionally related to Bcl-2 as well. It would be of interest to investigate more complex correlations between the levels of all these apoptosis stakeholders and response of the patients. This would allow simplification of the design of clinical trials as the number of patients will be reduced if they have been selected for expressing the appropriate target. To that purpose, surrogate tissues, such as PBMCs, that represent the target of innovative therapy strategies are required to select the patients. Unfortunately, we did not obtain any usable RNA from these samples. This indicates a problem in the sampling procedures that should be better evaluated, in real conditions, before any experiment. The extended goal is a 'proof of concept' for implementing pharmacogenomics/pharmacogenetics aspects into clinical trials. This study should be considered as a pilot study for the application of molecular biology and genetic technologies to improve the efficacy of cytotoxic therapy in a targeted therapy approach. We have demonstrated the feasibility of obtaining RNAs of good quality from tumor biopsies and of using microfluidic QPCR to profile these samples. Oblimersen up to a dose of 7 mg/kg/day given as a 24-h infusion on days 1–7 is feasible and can be safely administered in combination with standard TAC regimen on day 5 as neoadjuvant systemic therapy in patients with primary breast cancer [14]. However, there is room for improvement at both technical and logistic levels.

funding

acknowledgements

references

1. Kaufmann M, Hortobagyi GN, Goldhirsch A et al. Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: an update. J Clin Oncol 2006; 24: 1940–1949.

2. Reed JC. Mechanisms of apoptosis avoidance in cancer. Curr Opin Oncol 1999; 11: 68–75.

3. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood 1993; 81: 151–157.

4. Reed JC. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. Hematol Oncol Clin North Am 1995; 9: 451–473.

5. Berardo MD, Elledge RM, de Moor C et al. bcl-2 and apoptosis in lymph node positive breast carcinoma. Cancer 1998; 82: 1296–1302.

6. Webb A, Cunningham D, Cotter F et al. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. Lancet 1997; 349: 1137–1141.

7. van de Donk NW, Kamphuis MM, van Dijk M et al. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. Leukemia 2003; 17: 211–219.

8. Shangary S, Johnson DE. Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. Leukemia 2003; 17: 1470–1481.

9. Chi KN, Murrary RN, Gleave ME et al. A phase II study of oblimersen sodium (G3139) and docetaxel (D) in patients (pts) with metastatic hormone-refractory prostate cancer (HRPC). Proc Am Soc Clin Oncol 2003; 22 (Abstr 393).

10. Klasa RJ, Bally MB, Ng R et al. Eradication of human non-Hodgkin's lymphoma in SCID mice by BCL-2 antisense oligonucleotides combined with low-dose cyclophosphamide. Clin Cancer Res 2000; 6: 2492–2500.

11. Klasa RJ, List AF, Cheson BD. Rational approaches to design of therapeutics targeting molecular markers. Hematology Am Soc Hematol Educ Program 2001; 2001: 443–462.

12. Lopes de Menezes DE, Hudon N, McIntosh N, Mayer LD. Molecular and pharmacokinetic properties associated with the therapeutics of bcl-2 antisense oligonucleotide G3139 combined with free and liposomal doxorubicin. Clin Cancer Res 2000; 6: 2891–2902.

13. Vrignaud P, Lejeune P, Klem R, Bissery M-C. Combination of G3139 genasense™, a bcl-2 antisense oligonukleotide, with docetaxel (taxotere (R)) is active in a murine xenograft model of human non-small cell lung cancer. Proc Am Assoc Cancer Res 2002; 43: 578.

14. Rom J, von Minckwitz G, Eiermann W et al. Oblimersen combined with docetaxel, adriamycin and cyclophosphamide as neo-adjuvant systemic treatment in primary breast cancer: final results of a multicentric phase I study. Ann Oncol 2008; 19: 1698–1705.

15. Rodriguez C, Hughes-Davies L, Valles H et al. Amplification of the BRCA2 pathway gene EMSY in sporadic breast cancer is related to negative outcome. Clin Cancer Res 2004; 10: 5785–5791.

16. ten Asbroek AL, van Groenigen M, Nooij M, Baas F. The involvement of human ribonucleases H1 and H2 in the variation of response of cells to antisense phosphorothioate oligonucleotides. Eur J Biochem 2002; 269: 583–592.

17. Reed JC, Kitada S, Takayama S, Miyashita T. Regulation of chemoresistance by the bcl-2 oncoprotein in non-Hodgkin's lymphoma and lymphocytic leukemia cell lines. Ann Oncol 1994; 5 (Suppl 1): 61–65.

18. Schmitt CA, Rosenthal CT, Lowe SW. Genetic analysis of chemoresistance in primary murine lymphomas. Nat Med 2000; 6: 1029–1035.

19. Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol 1999; 17: 2941–2953.

20. Hersey P, Zhang XD. Overcoming resistance of cancer cells to apoptosis. J Cell Physiol 2003; 196: 9–18.

21. Jansen B, Schlagbauer-Wadl H, Brown BD et al. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat Med 1998; 4: 232–234.

22. Teixeira C, Reed JC, Pratt MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res 1995; 55: 3902–3907.

23. Yang D, Ling Y, Almazan M et al. Tumor regression of human breast carcinomas by combination therapy of anti-bcl-2 antisense oligonucleotide and chemotherapeutic drugs. Proc Am Assoc Cancer Res 1999; 40: 729.

24. Tolcher A, Miyake H, Gleave ME. Down-regulation of Bcl-2 expression by antisense oligonucleotide (AS-ODN) treatment enhances mitoxantrone cytotoxicity in the androgen-dependent Shionogi tumor model. Proc Am Assoc Cancer Res 1999; 40: 3198.

25. Miyake H, Tolcher A, Gleave ME. Antisense Bcl-2 oligodeoxynucleotides inhibit progression to androgen-independence after castration in the Shionogi tumor model. Cancer Res 1999; 59: 4030–4034.

26. Leonard JP, Coleman M, Vose J et al. Phase II study of oblimersen sodium (G3139) alone and with R-CHOP in mantle cell lymphoma (MCL). Proc Am Soc Clin Oncol 2003; 22: 631.

27. Morris MJ, Tong WP, Cordon-Cardo C et al. Phase I trial of BCL-2 antisense oligonucleotide (G3139) administered by continuous intravenous infusion in patients with advanced cancer. Clin Cancer Res 2002; 8: 679–683.

28. de Bono JS, Rowinsky EK, Kuhn J et al. Phase I pharmacokinetic (PK) and pharmacodynamic (PD) trial of bcl-2 antisense (Genasense) and docetaxel (D) in hormone refractory prostate cancer. Proc Am Soc Clin Oncol 2001; 20: 81.

29. Jansen BWV, Heere-Ress E, Hoeller C et al. Clinical, pharmacologic, and pharmacodynamic study of genasense (G3139, Bcl-2 antisense oligonucleotide) and dacarbazine (DTIC) in patients with malignant melanoma. Proc Am Soc Clin Oncol 2001; 20: (Abstr 1426).

30. Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002; 20: 709–760.

31. Zhao Q, Song X, Waldschmidt T et al. Oligonucleotide uptake in human hematopoietic cells is increased in leukemia and is related to cellular activation. Blood 1996; 88: 1788–1795.

32. Chang JC, Wooten EC, Tsimelzon A et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet 2003; 362: 362–369.