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Enhanced antitumour efficacy of gimatecan in combination with Bcl-2 antisense oligonucleotide in human melanoma xenografts

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Abstract

The anti-apoptotic protein Bcl-2 has been implicated in the intrinsic resistance of melanoma to chemotherapy. The aim of this study was to investigate the effects of anti-Bcl-2 oligonucleotide oblimersen on the antitumour activity of gimatecan, a novel lipophilic camptothecin currently undergoing clinical phase II studies. Results showed a reduced sensitivity of melanoma cells to gimatecan following *Bcl-2* transfection and inversely, increased cell sensitivity to gimatecan in combination with oblimersen. In *in vivo* studies performed in two melanoma xenografts expressing different Bcl-2 levels, the antitumour activity of oblimersen itself was modest, but the combination with gimatecan produced a significant therapeutic advantage. The combination therapy inhibited tumour growth and delayed regrowth of the two tumours tested. The enhancement of antitumour activity was observed at doses that were tolerated well. The effects of oblimersen on antitumour activity and toxicity of gimatecan were dose-dependent. The capability of oblimersen to improve the efficacy of gimatecan supports the therapeutic potential of the drug combination in the treatment of human melanoma.
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Keywords: Melanoma; Gimatecan; Bcl-2; Oblimersen

1. Introduction

Metastatic or recurrent melanoma is still an incurable disease. Although conventional chemotherapy, biological response modifiers such as interferons or interleukin-2 and vaccines have been recently shown to induce tumour response in melanoma patients, cure rates and survival are usually poorly affected by therapeutic approaches [1–3].

The treatment failure in metastatic melanoma reflects resistance to apoptotic stimuli [4]. However, mechanisms of drug resistance are often multifactorial and may include reduced drug accumulation through an increased efflux of drug associated with expression of ATP-binding cassette transporter proteins. Increased drug detoxification and DNA repair are also possible [4]. Defects in apoptosis signaling pathways have been implicated in the mechanisms of drug resistance in melanoma [5]. In particular, the expression of the anti-apoptotic protein Bcl-2 and preventing triggering of cell death has been suggested to play a role in modulating sensitivity to different classes of anticancer drugs in several tumour cell systems including melanomas [6–8].

Oblimersen is an antisense oligonucleotide that binds the bcl-2 mRNA sequence that results in the

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degradation of bcl-2 mRNA and down-regulation of Bcl-2 protein [9]. Preclinical and clinical studies have suggested the therapeutic potential of oblimersen combined with several cytotoxic agents against a variety of solid and hematologic malignancies [10,11]. In particular, a clinical study of the dacarbazine/oblimersen combination supported beneficial outcomes in melanoma patients receiving the combination in terms of progression-free survival [12].

Among the most clinically relevant antitumour drugs are camptothecins. They have a unique mechanism of action and a wide spectrum of antitumour activity [13]. Recently, a novel series of camptothecin analogues, substituted with lipophilic chains at position 7, was developed and the compound ST1481 (gimatecan) has selected on the basis of its cytotoxic potency and persistent topoisomerase-inhibitory activity [14]. Gimatecan delivered orally showed an impressive efficacy against a large panel of human tumours subcutaneously xenografted in nude mice. In particular, good antitumour activity was achieved on melanoma models [15]. The new camptothecin is endowed with high lipophilicity, lack of recognition by transporter proteins as demonstrated by *in vitro* and *in vivo* models and showed favourable pharmacokinetic behavior after oral delivery in mice [16,17]. Based on these promising preclinical features, gimatecan is currently in Phase I/II clinical studies.

The aim of this study was to investigate the effects of bcl-2 antisense oligonucleotide oblimersen on the antitumour activity of oral gimatecan against two human melanomas expressing different levels of Bcl-2. The results obtained showed that oblimersen significantly improved the efficacy of gimatecan against both melanomas and supports the pharmacological potential of the drug combination.

2. Materials and methods

2.1. Drugs and oligonucleotides

Gimatecan (ST1481, 7-*t*-butoxyiminomethylcamptothecin, Sigma-Tau, Pomezia, Rome) was dissolved in dimethylsulphoxide (DMSO) and stored at −20 °C until use. At treatment days, the drug was thawed and suspended in sterile, distilled water (DMSO 10% final concentration for *in vivo* injection). Oblimersen (G3139), a 18-mer phosphorothioate oligonucleotide (5′-TCTCCCAGCGTGCGCCAT-3′) directed against human bcl-2 mRNA, was provided dissolved in saline (Genta Inc., Lexington, MA, USA) and was further diluted in saline. The G3622 oligonucleotide, a reversed G3139 sequence control and 6-fluorescein labeled-G3139 oligonucleotide were provided by Genta Inc. as dried powder, that was freshly dissolved in saline. The phosphorothioated synthetic CpG oligonucleotide 1826 (CpG-ODN1826) was purchased from Coley Pharmaceutical Group (Kanata, Canada). In *in vivo* studies, all agents were administered in a volume of 10 ml/kg.

2.2. Cell lines and in vitro experiments

The human melanoma cell lines used in the study were grown in RPMI-1640 medium plus 10% fetal calf serum (Invitrogen, San Giuliano Milanese, Italy). The Me26414, Me10249, Me15392 cell lines were recently derived from metastatic melanoma lesions at the Istituto Nazionale Tumori. The 501Mel and LP cell lines were described previously [18,19]. The Bcl-2 overexpressing (MB6) and the empty vector-transfected (MN8) clones were derived from M14 cells, as reported [20].

Cellular sensitivity to gimatecan in MB6, MN8, M14 cells was measured by colony forming assay. Exponentially growing cells were exposed to the drug for 1 h. At the end of the treatment, the drug was replaced with fresh medium. Seventy-two hours after the end of treatment, cells were washed, assayed for cell viability (trypan blue exclusion test) and counted (Coulter Counter, Kontron Instruments, Milan, Italy). To evaluate the cell colony-forming ability, cell suspensions from different samples were seeded into 60-mm Petri dishes (Nunc, Mascia Brunelli, Milan, Italy) for 10 days. Colonies were stained with 2% methylene blue in 95% ethanol and counted (1 colony > 50 cells). The surviving fractions were calculated as the ratio of absolute survival of the treated sample/survival of untreated control sample. All experiments were repeated four times in triplicate.

Cell sensitivity to gimatecan in 501Mel and Me26414 cells and drug combination studies on Me26414 cells were performed using the cell growth-inhibition assay and percent of cell growth was assessed 72 h after treatment. Briefly, cells were seeded in 6-well plates and 24 h later exposed for 1 h to different concentrations of gimatecan. In combination studies, cells were then incubated in fresh medium and oblimersen (400 nM) was added in the presence of oligofectamine (2 μl/ml, Invitrogen).

Immunofluorescence analysis was performed to assess the cellular uptake of the fluorescein-labeled bcl-2 oligonucleotide (1 μM). Cells were seeded on cover glasses in 24-well plates and allowed to grow for 2 days. Cells were then treated with vehicle/oligonucleotide mix for 6 h. Oligofectamine was used as the vehicle for fluorescein-labelled bcl-2 oligonucleotide. For analysis of cellular uptake, glasses were mounted using standard techniques and viewed using a fluorescence microscope. Control slides included cells treated with vehicle or oligonucleotide alone.

Western blot analysis for Bcl-2 expression was carried out as previously described [21]. Briefly, samples were separated by SDS–PAGE and blotted onto nitrocellulose sheets. Blots were pre-blocked in phosphate-

buffered saline containing 5% (w/v) dried skimmed milk and then incubated overnight at 4 °C with an anti-Bcl-2 monoclonal antibody (1:100; Dako, Glostrup, Denmark). A rabbit anti-actin (1:100; Sigma Chemicals Co., St. Louis, MO, USA) or a mouse anti-HSP70/72 antibody (1:100; Oncogene Science Inc., Manhasset, NY, USA) was used as control for sample loading. The Western blots were developed by chemiluminescence procedures (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) to visualise protein presence. The expression level of Bcl-2 relative to actin was measured using ImageQuant (Molecular Dynamics, Sunnyvale, CA). The capability of oblimersen to down-regulate Bcl-2 expression was assessed by Western blot analysis after 24, 48 or 72 h incubation with oblimersen (400 nM) or the reversed sequence control oligonucleotide G3622 (400 nM) in the presence of oligofectamine (2 μl/ml).

2.3. In vivo experiments

All experiments were carried out using female athymic Swiss nude mice, 8–10 weeks-old (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms, where temperature and humidity were kept constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines.

For drug activity studies, exponentially growing 501Mel and Me26414 melanoma cells were subcutaneously (s.c) injected into the right flank of athymic nude mice (10^7 cells/mouse). Tumour lines were achieved by serial s.c. passages of tumour fragments (about $2 \times 2 \times 6$ mm) in healthy mice, as previously described [22]. Experiments were conducted in groups of four/six mice bearing bilateral s.c. tumours. Tumour fragments were implanted on day 0 and tumour growth was followed by biweekly measurements of tumour diameters with a Vernier caliper. Tumour volume (TV) was calculated according to the formula:

$$\mathrm{TV}\ (\mathrm{mm}^3) = d^2 \times D/2,$$

where d and D are the shortest and the longest diameter, respectively. Treatments started when tumours were just palpable and measurable ($\sim$50 mm^3). Oblimersen was delivered daily every morning by intraperitoneal (i.p.) or intravenous (i.v.) injections. Gimatecan was given orally in the afternoon employing courses of 5 daily administrations per week.

The efficacy of the drug treatment was assessed by:

(i) Tumour volume inhibition percentage (TVI%) in treated *vs.* control mice, calculated as: TVI% = 100 − (mean TV treated/mean TV control × 100).
(ii) Gross $\log_{10}$ cell kill (LCK) calculated by the formula: LCK = (T − C)/3.32 × DT, where T and C are the mean times (days) required for treated (T) and control (C) tumours, respectively, to reach an established TV and DT is the doubling time of control tumours.
(iii) Complete response (CR) i.e., total disappearance of tumour lasting at least 10 days.

The toxicity of the drug treatment was defined by body weight loss (BWL) and lethal toxicity. The highest body weight loss percentage induced by treatments is reported in Tables 2 and 3. Deaths occurring in treated mice before the death of the first control mouse as well as late deaths of tumour-free mice were ascribed to toxic effects. Persistent body weight loss in untreated control mice, bearing established tumours, was considered a cachectic effect induced by tumours.

To assess non-antisense effects of oblimersen *in vivo*, the production of selected cytokines was analysed. The CpG-ODN1826, a nucleotide where the sequence has been optimised for potent modulation of innate immunity, was used as positive control. Healthy nude mice received i.v. or i.p. injection of oblimersen or CpG-ODN1826 (10 mg/kg). Two hours later, under light anesthesia, mice were bled from the retro-orbital sinus and killed by cervical dislocation. Interleukin-12 (IL-12) production was analysed in supernatants from spleen cell suspensions incubated in 24-well plates (10^7 cell/well) for 18 h in complete RPMI supplemented with 10% fetal calf serum [23]. Levels of keratinocyte-derived chemokine (KC), a functional homologue of human interleukin-8 (IL-8), were analysed in serum samples. Quantification of IL-12 in supernatants and KC in sera was determined using enzyme-linked immunosorbent assay (ELISA) kits from BD PharMingen (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

Student's t test (two tailed) and Fisher's exact test were used for statistical comparison of tumour volumes and CR rates, respectively.

3. Results

3.1. Influence of Bcl-2 expression on gimatecan cytotoxicity

To determine if the sensitivity of human melanoma to drug might be related to the level of Bcl-2 expression, we compared the cytotoxic effects of gimatecan in human melanoma M14 cells transfected with vector containing *Bcl-2* (MB6 clone) to those transfected with the empty vector (MN8 clone). The expression level of Bcl-2 in each cell line is shown in Fig. 1(a). As expected, MB6 cells had higher levels of Bcl-2 protein compared to

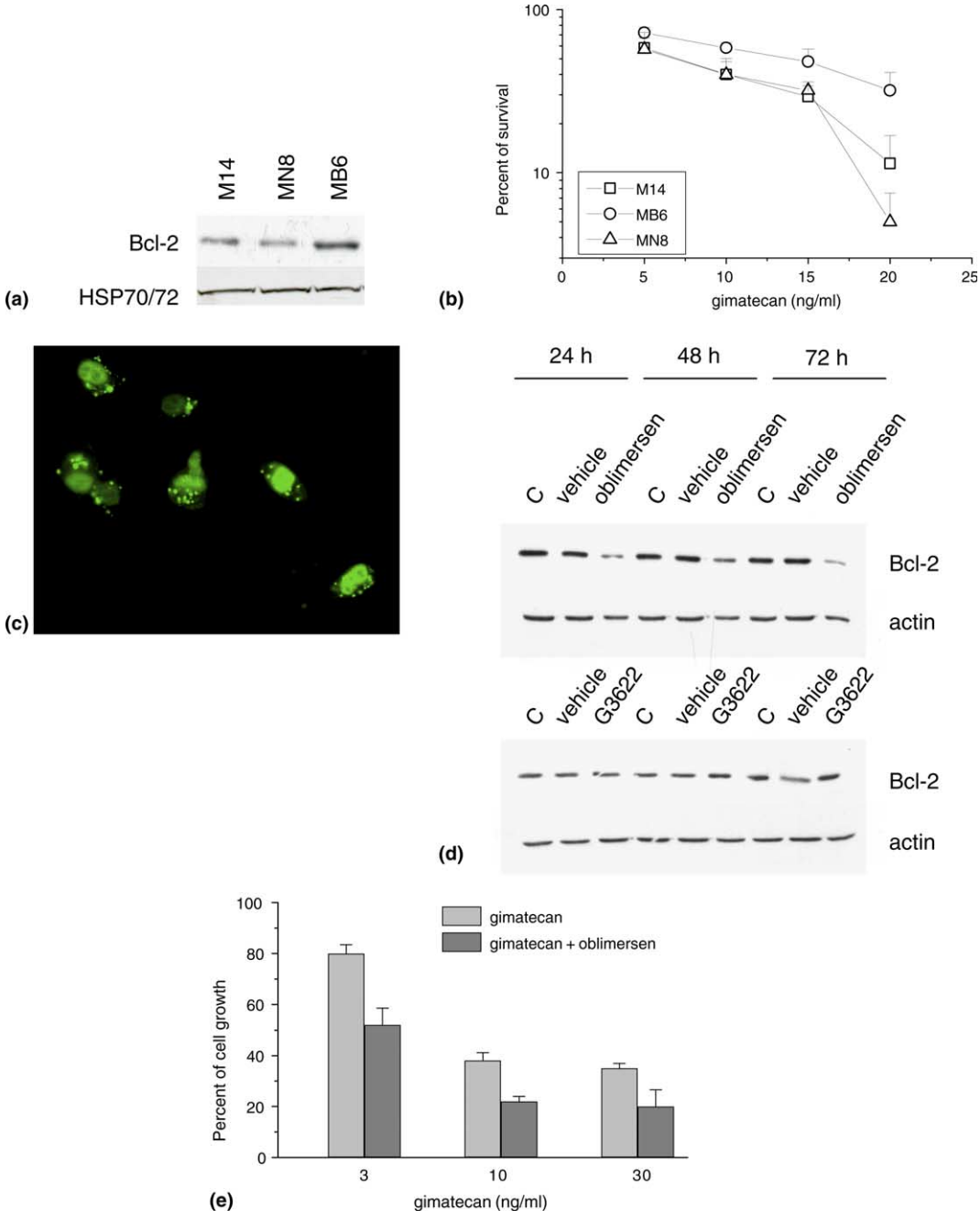

Fig. 1. Influence of Bcl-2 level on melanoma cell sensitivity to gimatecan. (a) Western blot analysis of Bcl-2 protein levels in parental cells (M14), in a control transfectant clone (MN8), and in a bcl-2 transfected clone (MB6). Control loading is shown by HSP70/72. (b) Cell survival (by clonogenic assay) to gimatecan exposure (1 h) in M14, parental cell line (□); MB6, Bcl-2 overexpressing clone (○); MN8, empty-vector transfected clone (△). (c) Cellular uptake of fluorescein-labelled anti-Bcl-2 oligonucleotide. Cells were observed 6 h after exposure to 1 μM oligonucleotide in the presence of oligofectamine (vehicle). (d) Western blot analysis of Bcl-2 expression after exposure of Me26414 cells to oblimersen (G3139) or to control G3622 oligonucleotide (400 nM for both). (e) Effect of oblimersen on Me26414 cell sensitivity (by growth-inhibition assay) to gimatecan. Cells were exposed to gimatecan (1 h) and then incubated with 400 nM oblimersen in the presence of oligofectamine (72 h).

the other clone. Survival of M14 cells after 1 h-exposure to a range of gimatecan concentrations was determined by the clonogenic test. The Bcl-2 overexpressing cells (MB6) exhibited reduced sensitivity to the agent compared with empty-vector transfected (MN8) and untransfected M14 cells (Fig. 1(b)).

The possibility of sensitising melanoma cells to gimatecan by using oblimersen was explored using the Me26414 cell line. Fluorescent microscopy analysis indicated that in the cells used, exposure to oblimersen in the presence of the carrier oligofectamine resulted in intracellular uptake of the fluorescein-labelled oligonucleo-

tide. This was shown by bright intranuclear and cytoplasmic fluorescence (Fig. 1(c)). Fluorescence was not seen in control cells treated with oligonucleotide alone (data not shown). The capability of oblimersen to down-regulate Bcl-2 expression was assessed by Western blot analysis in cells exposed for 24, 48 or 72 h to 400 nM oblimersen or to the reverse sequence control oligonucleotide G3622 in the presence of oligofectamine (Fig. 1(d)). A marked decrease of the expression of Bcl-2 was observed as soon as 24 h after exposure to oblimersen, whereas G3622 exposure did not affect Bcl-2 levels. Growth-inhibition assays indicated that when cells were exposed to the combination of gimatecan (1 h exposure) and oblimersen (400 nM, added for 72 h at the end of gimatecan exposure with vehicle), increased growth inhibition was seen (Fig. 1(e)). Under these conditions, the oligonucleotide alone produced marginal effects on cell growth (<20%) and oligofectamine did not modify cell sensitivity to gimatecan (data not shown).

3.2. In vivo antitumour activity studies

The expression levels of Bcl-2 in different melanoma cell lines were examined by Western blot analysis (Fig. 2). The Bcl-2 protein was detected in all five cell systems with variable levels of protein expression. 501Mel and LP cells had the highest level while Me26414 and Me10249 cells had the lowest expression levels.

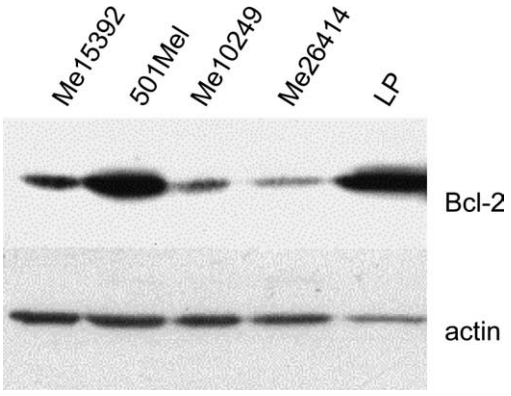

Fig. 2. Western blot analysis of Bcl-2 levels in different human melanoma cell lines. Control for loading is shown by actin.

On the basis of different expression levels of Bcl-2 and capability to grow as tumour xenografts, the 501Mel (high Bcl-2 level) and Me26414 (low Bcl-2 level) tumour models were chosen for antitumour activity studies. Features of the two melanomas are summarised in Table 1. Both melanoma models were tumourigenic following s.c. cell inoculum in nude mice (tumour take >95%) with different growth patterns. In contrast to the Me26414 model, the 501Mel melanoma grew slowly (D.T., 9.1 days) with melanotic pigmentation and non-lethal cachexia (BWL, 10%). Since both tumours were characterised by wild-type p53, it is unlikely that the differential expression of Bcl-2 was related to *p53* gene. An analysis of cell sensitivity to gimatecan indicated that 501Mel cells were around 6 times more resistant to gimatecan than Me26414 cells with a higher IC_{50} value (Table 1).

In experiments with 501Mel melanoma, one dose level of oblimersen (10 mg/kg) was tested according to two schedules and routes of administration (Table 2). Oblimersen alone, delivered i.p. daily for five days a week (daily × 5/week) for three weeks, achieved a marginal effect. Camptothecin alone at a suboptimal regimen (0.25 mg/kg, daily × 5/week, every other week for 2 courses) induced, as expected, a low inhibition of tumour growth (TVI 44%) considering the discontinuous and short administration schedule employed. The camptothecin alone was well tolerated, whereas mice treated with the combination, exhibited substantial BWL without toxic death. When gimatecan, according to the suboptimal regimen, was combined with oblimersen, the antitumour activity significantly increased compared to that achieved by the drug alone (TVI 80%, $P < 0.01$) despite the negligible effect of the antisense oligonucleotide itself on tumour growth (Fig. 3(a)). Thus, oblimersen was able to significantly enhance the antitumour effects of a suboptimal gimatecan treatment.

Using the optimal schedule (daily × 5/week for 4 weeks), the 501Mel melanoma was very responsive to the prolonged treatment with gimatecan (Table 2). Antitumour efficacy achieved by the combination with the antisense (i.v., daily × 3/week for 4 weeks) was similar to that produced by gimatecan alone in terms of TVI

Table 1
Features of the human melanoma models employed in the study

Model	Cellular features			*In vivo* s.c. tumour		
	p53 status	Bcl-2 level	Gimatecan IC_{50}[a] (ng/ml)	DT[b]	Pigmentation	Cachexia[c]
501Mel	Wild-type	High	48.3 ± 3.0	9.1	Melanotic	10
Me26414	Wild-type	Low	7.6 ± 0.7	4.5	Amelanotic	0

[a] Cell sensitivity to gimatecan was assessed by growth-inhibition assay after 1 h drug exposure. Values are the mean (±SD) of 4 independent experiments. IC_{50}, drug concentration inhibiting 50% of cell growth.
[b] Tumour Doubling Time (days) obtained from semilog best-fit curves of mean tumour volumes in untreated control mice plotted against time.
[c] Body weight loss % in untreated control mice; the highest value is reported.

Table 2
Effects of the Bcl-2 antisense oligonucleotide oblimersen on the antitumour activity of oral gimatecan in nude mice s.c. bearing the 501Mel human melanoma

Drug[a]	Days of treatment	TVI% at day[b]			LCK[c]	BWL%[d]	Tox[e]
		36	40	80			
Oblimersen i.p., 10 mg/kg	6 → 10, 13 → 17, 20 → 24	18			0.1	10 (50)	0/5
Gimatecan, 0.25 mg/kg	6 → 10, 20 → 24	44			0.4	3 (13)	0/5
Oblimersen + gimatecan		80*			0.7	12 (13)	0/5
Oblimersen i.v., 10 mg/kg	3 → 5, 10 → 12, 17 → 19, 24 → 26		37	0	0.3	10 (59)	1/4 (40)
Gimatecan, 0.25 mg/kg	3 → 7, 10 → 14, 17 → 21, 24 → 28		96	74	1.2	8 (10)	0/4
Oblimersen + gimatecan			96	98	2.6	17 (7)	0/4

[a] Oblimersen was delivered 4–5 h before gimatecan.
[b] Tumour volume inhibition % in treated over control mice.
[c] Gross $\log_{10}$ kill to reach 300 mm^3 of tumour volume.
[d] Body weight loss %. The highest change is shown. In parentheses the day on which it was observed.
[e] Dead mice/treated mice; in parentheses the day of death.
* $P < 0.01$ by Student's t test *vs.* gimatecan-treated tumours.

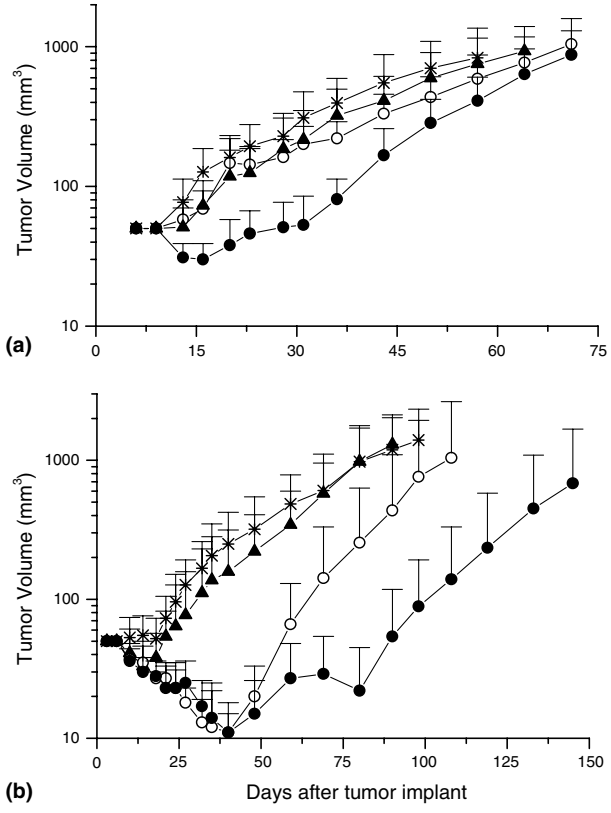

Fig. 3. Effect on tumour growth of oblimersen and oral gimatecan in 501Mel human melanoma xenografts. (a) ✻, control tumours; ▲, i.p. oblimersen, 10 mg/kg, daily for 5 days/week for 3 weeks; ○, gimatecan, 0.25 mg/kg, daily for 5 days/week every other week for 2 courses; ●, oblimersen plus gimatecan, delivered as above. Treatment started at day 6. (b) ✻, control tumours; ▲, i.v. oblimersen, 10 mg/kg, daily for 3 days/week for 4 weeks; ○, gimatecan, 0.25 mg/kg, daily for 5 days/week for 4 weeks; ●, oblimersen plus gimatecan, delivered as above. Treatment started at day 3. Each point represents the mean tumour volume from 8–10 tumours. Bars represent S.D.

(TVI 96% for both groups on day 40). However, the antitumour effect achieved by the combination was much more lasting over time (Fig. 3(b)). In fact, 50 days after the last treatment, i.e., day 80, tumours were still inhibited (TVI 98%) in the group receiving the combination, whereas in mice treated with gimatecan alone the tumours had already regrown (TVI 74%). The persistent inhibitory effect of the combination was reflected in a markedly higher LCK value (2.6 *vs.* 1.2). The antisense oligonucleotide alone induced a marginal tumour growth inhibition ($P > 0.05$ *vs.* control tumours) with an appreciable body weight loss (10%) and one toxic death. An increased body weight loss was observed in the combination-treated compared to the gimatecan-treated mice (17 *vs.* 8%), without toxic deaths.

Regarding treatment toxicity, marginal changes on body weight were produced by single agent treatment during the treatment period (Table 2), whereas more than 10% BWL was observed in mice receiving combination therapy; however, all mice recovered and no toxic deaths were observed. In 501Mel-bearing mice treated with oblimersen alone, 10% BWL was observed only at advanced stage of tumour growth. This change was likely related to tumour–host interaction as untreated control mice presented 10% BWL before being sacrificed for tumour burden (Table 1).

The effects of Bcl-2 antisense oligonucleotide on the antitumour activity of oral gimatecan against the Me26414 melanoma are shown in Table 3 and Fig. 4. A range of doses of oblimersen (5, 7.5 and 10 mg/kg) was investigated delivering the drug i.v. daily × 3/week for 4 weeks. In treated animals, tumour growth was significantly reduced compared to the controls, even though the effects of oblimersen were not dose-dependent, both in terms of TVI (42–55%, $P < 0.05$ *vs.* control tumours for all groups) or LCK (0.7–1). No toxicity was observed. Gimatecan, 0.25 mg/kg, delivered by the optimal treatment schedule (daily × 5/week for 4 weeks) was very effective in inhibiting tumour growth, inducing a TVI of 97% and a LCK of 3.2, without severe toxicity. When the two agents were combined, using the low dose of oblimersen (5 mg/kg), a significant enhancement of

Table 3
Effects of the i.v. Bcl-2 antisense oligonucleotide oblimersen on the antitumour activity of oral gimatecan in nude mice s.c. bearing the Me26414 human melanoma

Drug[a]	Dose (mg/kg)	Days of treatment	TVI%[b]	CR[c]	NED[d]	LCK[e]	BWL%[f]	Tox[g]
Oblimersen	5	3 → 5, 10 → 12, 17 → 19, 24 → 26	51	0/10	–	1	0	0/5
	7.5		42	0/10	–	0.7	0	0/5
	10		55	0/12	–	0.9	0	0/6
Gimatecan	0.25	3 → 7, 10 → 14, 17 → 21, 24 → 28	97	0/10	–	3.2	9 (28)	0/5
Oblimersen + gimatecan	5	3 → 5, 10 → 12, 17 → 19, 24 → 26						
	0.25	3 → 7, 10 → 14, 17 → 21, 24 → 28	99*	6/12**	0/8	3.6	15 (28)	2/6 (50, 76)
Oblimersen + gimatecan	7.5	3 → 5, 10 → 12, 17 → 19, 24 → 26						
	0.25	3 → 7, 17 → 21	99*	6/12**	4/12 (120)	4.2	14 (10)	0/6
Oblimersen + gimatecan	10	3 → 5, 10 → 12						
	0.25	3 → 7, 10 → 14	–	4/ 4	4/4 (120)	–	11 (14)	4/6 (17,17,17,18)

[a] Oblimersen was delivered 4–5 h before gimatecan.
[b] Tumour volume inhibition % in treated over control mice, assessed 35 days after tumour injection.
[c] CR, Complete Response, i.e., disappearance of tumour for at least 10 days.
[d] NED, no evidence of disease at the end of the experiment. In parenthesis the day on which it was observed.
[e] Gross log10 cell kill to reach 1000 mm^3 of tumour volume.
[f] Body weight loss %. The highest change is shown. In parentheses the day on which it was observed.
[g] Dead mice/treated mice; in parentheses the days of death.
* $P < 0.000001$ by Student's t test, *vs.* gimatecan-treated tumours.
** $P < 0.05$ by Fisher's exact text *vs.* gimatecan-treated tumours.

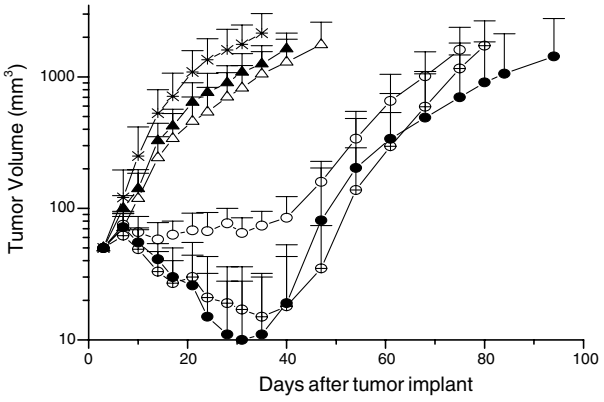

Fig. 4. Effect on tumour growth of i.v. oblimersen and oral gimatecan in Me26414 human melanoma xenografts. Treatment started at day 3. (∗), control tumours; oblimersen, (△) 5 mg/kg, and (▲) 7.5 mg/kg, daily for 3 days/week for 4 weeks; (○), gimatecan, 0.25 mg/kg, daily for 5 days/week for 4 weeks; (⊕), oblimersen, 5 mg/kg, plus gimatecan, 0.25 mg/kg, given as above; (●), oblimersen, 7.5 mg/kg, as above, plus gimatecan, 0.25 mg/kg, daily for 5 days/week every other week for 2 courses. Each point represents the mean tumour volume from 10–12 tumours. Bars represent S.D.

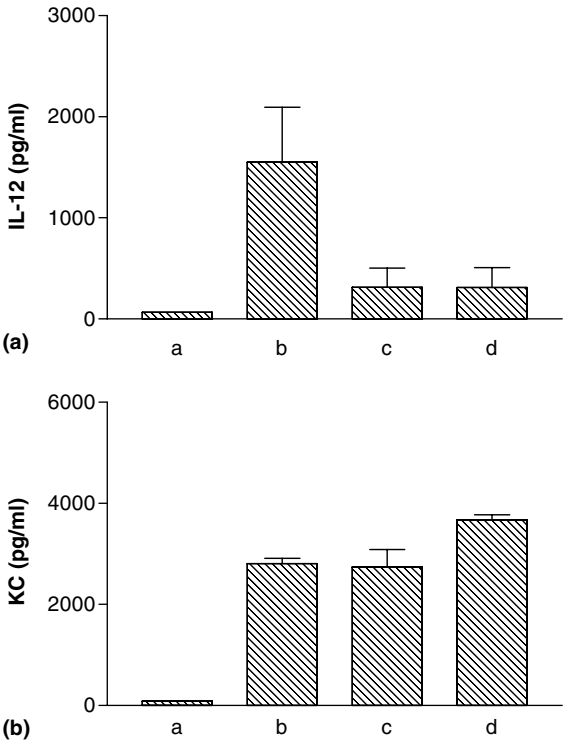

Fig. 5. Levels of interleukin 12 (IL-12) and keratinocite-derived (KC) chemokine evaluated by enzyme-linked immuno assay. (a) Concentration of IL-12 in supernatants of spleen cells from athymic nude mice. (b) Concentration of KC from athymic nude mice serum. Symbols a – untreated; b – treated with CpG-ODN1826, 10 mg/kg i.v.; c – treated with oblimersen, 10 mg/kg i.v.; d – treated with oblimersen, 10 mg/kg i.p. Mice were sacrificed 2 h after treatment. Bars represent S.D.

antitumour effects could be achieved in terms of TVI (99 *vs.* 97%, $P < 0.000001$ compared to gimatecan-treated mice), LCK (3.6 *vs.* 3.2) and CR (6/12 *vs.* 0/10, $P < 0.05$). However, toxic manifestations were also enhanced in the combination-treated mice as two mice died of toxicity. When a higher dose of oblimersen (7.5 mg/kg) was combined with gimatecan only two courses of gimatecan (daily × 5/week, intervalled by 10 days) could be tolerated. In spite of the reduced dose of gimatecan, an improvement of efficacy was achieved as the combination produced 6/12 complete regressions and 4 of them were long lasting (no evidence of disease at 120 days). Moreover, no toxic death occurred despite an appreciable BWL (14%). When gimatecan was combined with the highest dose of oblimersen (10 mg/kg)

four out six mice died for toxicity after two weeks of treatment. However, in surviving mice, tumours never regrew up to the end of the experiment (day 120).

Since oblimersen sequence contains CpG motifs and a potential contribution to modulate innate immunity is possible [24], we compared the effects of oblimersen and the immunostimulating CpG-ODN1826 on the production of selected chemokines. Oblimersen, induced a comparable stimulation of KC/IL-8 and a lower stimulation of IL-12, regardless of the route of administration (Fig. 5).

4. Discussion

The present study indicates that expression of Bcl-2 plays a role in determining sensitivity to the 7-substituted camptothecin gimatecan. Indeed, we found a reduced cytotoxicity of the drug in a melanoma clone overexpressing Bcl-2 as compared with the parental cell line. On the other hand, treatment with a Bcl-2 antisense increased the drug cytotoxicity. Based on the variable expression of Bcl-2 in human melanoma cells [25], we used two human melanoma xenografts generated from the 501Mel and Me26414 cell lines, characterized by differential Bcl-2 expression and by different sensitivities to gimatecan, to investigate the effects of oblimersen on the antitumour activity of gimatecan. The biological effects of oblimersen were somewhat different in the two models. High dose of oblimersen alone was not effective in inhibiting the growth of the Bcl-2 overexpressing 501Mel melanoma. The observed lethal toxicity in such a model was likely mediated by tumour–host interaction, i.e., by the property of the tumour to induce cachexia. In keeping with a lower Bcl-2 expression, the Me26414 melanoma xenograft was more sensitive to oblimersen than the 501Mel xenograft and the antitumour activity was not dose-related. Moreover, the treatment was not associated with any toxic manifestation.

Oblimersen was effective in increasing the antitumour effects of gimatecan in both melanoma xenografts. In the 501Mel melanoma xenograft, oblimersen could enhance the antitumour effects of an intermittent suboptimal regimen of gimatecan. In addition, the antitumour efficacy was substantially improved even when the camptothecin was administered with its optimal schedule of protracted daily treatment. In such conditions, the tumour growth was markedly inhibited and the regrowth was delayed. Indeed, in the group treated with the camptothecin alone, 3 out 8 tumours regrew 10 days after the treatment end, whereas in the combination-treated mice only 2/8 tumours regrew 50 days after the treatment end. The change in body weight in 501Mel tumour-bearing mice was a late event and did not account for the differential efficacy of the combination in the two models.

Using the same prolonged treatment schedule, gimatecan itself was very effective in inhibiting the growth of the Me26414 melanoma model, but no complete regressions were achieved by treatment with camptothecin alone. In such a model the therapeutic advantage of co-treatment with oblimersen could be documented by a complete disappearance of most treated tumours (16/28 complete regressions for at least 10 days) and by the occurrence of 8/24 persistent tumour regressions.

Although the antitumour effects of oblimersen itself were apparently not dose-dependent, both the antitumour activity and the toxicity of the combination were dependent on the dose of oblimersen (Table 3). Indeed, using the same daily dose of gimatecan (0.25 mg/kg), it is evident that the combination with oblimersen 10 mg/kg was substantially more toxic than 5 mg/kg, despite a lower cumulative dose of gimatecan. A comparison of the tolerance of the combination in animals treated with 7.5 and 10 mg/kg of oblimersen clearly indicated that the therapeutic index of the co-treatment was critically dependent on the schedule rather than on the single or cumulative dose of each component in the combination. A recovery interval between repeated courses with gimatecan could improve the tolerance of the combination. Indeed, such a schedule provided the best results in terms of tolerability and antitumour effects. Such findings are in keeping with our preclinical results with the combination of a CpG oligonucleotide (ODN1668) and another camptothecin (topotecan). In fact, the alternating sequence of the two therapies was markedly toxic, whereas the sequential schedule (full chemotherapy regimen followed by the CpG oligonucleotide) with the same doses of the two agents was well tolerated [23]. The ability of CpG oligonucleotides to stimulate extramedullary murine hematopoiesis through release of IL-8 from neutrophiles, macrophages and dendritic cells has been documented [26,27] and is possibly responsible for the enhancement of toxicity by combination with myelosuppressive drugs. Interestingly, our results clearly indicate the ability of oblimersen to strongly upregulate KC/IL-8 plasma levels. The increase was comparable to that achieved by CpG-ODN1826, the positive CpG oligonucleotide control.

The relative contribution of Bcl-2-independent effects of oblimersen in its antitumour activity is still controversial [10,28]. In our study, we showed that oblimersen has a low inducing effect of IL-12, as compared to the unmethylated CpG-ODN1826, a potent stimulator of innate immunity. Induction of IL-12, and not of IFNγ or TNF, was implicated in the increased antitumour effect achieved by the combination of a CpG oligonucleotide and topotecan [23]. Although an increased contribution of the putative immunostimulation is expected in syngenic mice tumours, in our experimental model stimulation of the immune system seems to play a marginal role in the increased antitumour response of the melanoma xenografts to gimatecan plus antisense Bcl-2. Such find-

ings are in keeping with other preclinical studies in perforin-knock-out (natural killer deficient) mice, in SCID mice or in nude mice [29–31].

An additional contribution to the antitumour effect of the combination could be related to the antiangiogenic potential of the drug. Indeed, a low daily dose of gimatecan has been reported to be associated with a marked reduction of microvessel density in a human tumour xenograft [32]. Relevant to this point is the observation that the antisense oligonucleotide itself may have antiangiogenic activity [33].

Under our experimental conditions, oblimersen produced down-regulation of the Bcl-2 protein (Fig. 1) but did not affect the level of Bcl-x_L (not shown). Moreover, we did not observe down-regulation of poly(ADP-ribose)polymerase, which has been implicated in the mechanism of action of oblimersen in prostate carcinoma cells [28]. Although these results and the lack of effect of the reverse sequence control oligonucleotide (G3622) support a specific modulation of the target, additional effects on signal-tranduction pathways of camptothecin-induced apoptosis involving Bcl-2 could not be ruled out. In particular, production of reactive oxygen species could contribute to the cytotoxic effect, because oxidative stress is implicated in cellular response to camptothecins [34].

In conclusion, gimatecan, a novel lipophilic camptothecin, exhibited an outstanding efficacy against this tumour type in preclinical setting, as already observed in other human melanoma models [15]. Using daily treatment schedules, the Bcl-2 antisense oligonucleotide was able to improve the efficacy of the camptothecin and to enhance the persistence of the tumour growth inhibition. Both agents are currently in clinical development. Our results support the therapeutic potential of oblimersen and gimatecan in combination for the treatment of melanoma.

Conflict of interest statement

None declared.

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