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Combination of a CpG-oligodeoxynucleotide and a topoisomerase I inhibitor in the therapy of human tumour xenografts

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Abstract

The study was conducted to investigate the effects of a novel therapeutic approach, i.e. the combination of chemotherapy and immunotherapy, against a human prostate carcinoma xenograft. A topoisomerase I inhibitor, topotecan, and CpG-containing oligodeoxynucleotides (CpG-ODN) were combined. Athymic mice bearing the PC-3 human prostate carcinoma were treated with the maximum tolerated dose (MTD) of topotecan (3 weekly treatments) and with repeated treatments of CpG-ODN (40 and 20 μg/mouse); tumour growth and lethal toxicity were monitored. Topotecan effect on CpG-ODN-induced production of interleukin (IL) 12, interferon (IFN)-γ and tumour necrosis factor-α was also assessed. Since topotecan pretreatment differentially influenced CpG-ODN-induced production of IL-12 and IFN-γ, the antitumour effects of the two therapies were investigated in a sequential (full topotecan regimen followed by CpG-ODN) or in an alternating sequence (starting with CpG-ODN). Topotecan inhibited PC-3 tumour growth, inducing 95% tumour volume inhibition. All combined treatments resulted in a significant delay in tumour growth, compared to the effects in topotecan-treated mice ($P < 0.01$, by analysis of tumour growth curves). The combination regimens were well tolerated, except for the alternating sequence of 40 μg CpG-ODN and topotecan, which resulted in three out of eight toxic deaths. This alternating sequence was highly toxic even when another cytotoxic drug (doxorubicin) was used in healthy mice. In conclusion, the combination of topotecan and CpG-ODN increased antitumour effects over chemotherapy alone in the growth of a human prostate carcinoma xenograft. Administration sequence was critical to the combination toxicity: the complete regimen of the cytotoxic drug followed by repeated administrations of the immunomodulator seemed the most promising for further investigations.

Keywords: Antitumour therapy; Topotecan; CpG-oligodeoxynucleotide; Combined therapies; Toxicity

1. Introduction

Many clinical trials in cancer patients have demonstrated the superior efficacy of the combination of different therapies as compared to the use of any single particular modality. However, few studies of combined immunotherapy and chemotherapy have been conducted, mainly because it is widely assumed that the immunosuppressive aspect of chemotherapy negates the benefits of immunotherapy.

Recently, cells of the innate immune system were found to express a set of pattern-recognition receptors that can detect certain molecular structures present in pathogens, but not in eukaryotic cells [1]. Bacterial DNA, for example, activates cells of the innate immune system due to the relative abundance of unmethylated CpG motifs in its DNA. These CpG motifs are suppressed and methylated in vertebrate DNA [2]. Synthetic oligodeoxynucleotides (ODN) with immunostimulatory CpG motifs mimic the effects of bacterial DNA [2]. Among pattern recognition receptors, the Toll-like receptors (TLRs) play a pivotal role [3]. Such a receptor, TLR-9, is essential for the activation of innate immune cells by CpG motifs [4]. The observation that ODN-containing

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CpG dinucleotides (CpG-ODN) exhibit several immunological effects has led to their use even in the field of oncology. In preclinical systems CpG-ODN have been found to be promising tools as adjuvants for tumour vaccines and as tumour immunotherapeutics, either alone or in combination with antitumour antibodies [5–10]. Like most immunotherapies, CpG-ODN treatment in animal models is most effective if administered when the tumour burden is small, or in the presence of minimal residual disease, as is the case following surgery or effective chemotherapy. The reported ability of CpG-ODN to protect B cells from apoptosis, induced by agents such as ultraviolet irradiation, vinca alkaloids and doxorubicin [11], might represent an advantage over other immunostimulators for combination with chemotherapy. Indeed, CpG-ODN acting only on haematological cells expressing TLR-9 would reduce the immunosuppressive effect without blocking the activity of chemotherapeutic drugs on solid tumours.

Camptothecins are well-established cytotoxic drugs that target DNA topoisomerase I, a critical enzyme for many DNA functions [12]. Topotecan is a water-soluble camptothecin that in experimental studies exhibits impressive antitumour activity against a large panel of human tumour xenografts [13], including hormone-refractory human prostate carcinoma xenografts [14]. The drug is currently in clinical use for lung and ovarian carcinomas, and its dose-limiting toxicity is neutropenia with or without thrombocytopenia [15].

In the present study, we investigated the therapeutic potential of combined cytotoxic and immunostimulating therapies on the growth of a human tumour xenograft in nude mice. The hormone-refractory PC-3 human tumour xenograft was chosen based on its high sensitivity to topotecan [14]. The chemotherapy treatment was designed in order to achieve a minimal residual disease condition. Analysis of tumour growth and toxicity in tumour-bearing mice treated with CpG-ODN, during or after the optimal therapeutic regimen of topotecan, indicated an advantage of the combination with respect to time of tumour regrowth and a critical role for treatment sequence in determining toxic effects.

2. Materials and methods

2.1. Drugs and synthetic ODNs

Topotecan (Hycamtin; Glaxo SmithKline S.p.A., Verona, Italy) and doxorubicin (Adriblastina; Pharmacia S.p.A., Milan, Italy) were suspended in sterile distilled water. Purified, single-stranded, phosphorothioated ODN 1668 (5′-TCCATGACGTTCCTGATGCT-3′) containing a CpG motif [16] was synthesised under endotoxin-free conditions by M-Medical-GENENCO (Firenze, Italy). Phosphorothioate modification was used in order to reduce the susceptibility of the ODN to DNAse digestion, thereby significantly prolonging its half-life *in vivo*. CpG-ODN was dissolved in sterile saline.

2.2. Tumour cells

PC-3 prostate carcinoma cells (from ATCC) were maintained in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) v/v (Life Technologies Inc., Gaithersburg, MD).

2.3. Mice

Male athymic Swiss nude and FVB mice (purchased from Charles River, Calco, Italy) were maintained in laminar-flow rooms at constant temperature and humidity, with food and water given ad libitum. Mice were used at 8–12 weeks of age (18–24 g body wt). Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan, according to UKCCCR guidelines [17].

2.4. In vivo studies

For antitumour activity studies, exponentially growing PC-3 cells were inoculated subcutaneously in the right flank ($8–10\times10^6$ cells) of male athymic nude mice. Each control or drug-treated group included eight mice. Tumour growth was followed by bi-weekly measurements of tumour diameters with a Vernier caliper. Tumour volume (TV) was calculated in mm^3 according to the formula: $TV = d^2\times D/2$, where d and D are the shortest and the longest diameter, respectively. Drug treatment started when mean TV was about 100 mm^3. Topotecan was delivered orally by gavage (in a volume of 10 ml/kg body wt) at a dose of 15 mg/kg, three times at 7-day intervals (q7d×3). CpG-ODN was delivered intraperitoneally (200 μl/mouse) at doses of 20 and 40 μg/mouse, every third/fourth day for seven times. In the combination groups, CpG-ODN was delivered before and during topotecan regimen (alternating schedule) or starting 7 days after the last topotecan treatment (sequential schedule) (see Table 2). Control mice were solvent-treated orally and intraperitoneally, in parallel with drug treatments.

The percentage of tumour volume inhibition (TVI) in treated versus control mice was calculated at different times and indicated as cTVI%. After control mice had been killed due to tumour burden, the TVI% was calculated in the combination-treated versus the topotecan-treated mice and indicated as tTVI%. Optimal cTVI% and tTVI% are reported in Table 2. Growth curves were designed, plotting mean TV versus time. Toxic effects of drug treatment were assessed as: (i) lethal toxicity, i.e. any death in treated mice before any

control mice, as monitored daily; (ii) percent of body weight loss induced by the treatment. Mice were weighed twice a week.

The toxicity experiments were performed using healthy euthymic FVB mice. Doxorubicin (7.5 mg/kg) was delivered intraperitoneally, q7d×3, and CpG-ODN (40 μg/mouse) was delivered intraperitoneally, q7d×3, starting 3 days before the first doxorubicin treatment (alternating schedule) or 7 days after the last doxorubicin injection (sequential schedule). Lethal toxicity was assessed.

2.5. Quantification of interferon-γ (IFN-γ), interleukin 12 (IL-12), and tumour necrosis factor-α (TNF-α) production

Healthy athymic nude mice were used for assessment of IFN-γ, IL-12 and TNF-α production after CpG-ODN treatment. Each experimental group consisted of three to six mice. Drugs were prepared as for antitumour activity studies. CpG-ODN was given intravenously at the dose of 200 μg/mouse; topotecan (15 mg/kg) was given once by gavage, followed by a single administration of CpG-ODN given 1, 3, 7 or 14 days later. Two hours after CpG-ODN injection, mice under a light anaesthesia were bled from the retro-orbital sinus and killed by cervical dislocation. Sera were analysed for TNF-α production. Spleens were aseptically removed, mechanically dissociated, and splenocytes (10^7/well) were cultured for 18 h in 24-well plates in 2 ml complete RPMI supplemented with 10% FCS v/v. Supernatants were analysed for IFN-γ and IL-12 (p40 form). All cytokines were assessed by enzyme-linked immunoassay (BD PharMingen, San Diego, CA).

2.6. Statistical analysis

Results of the antitumour experiments were statistically evaluated by analysis of variance (ANOVA) using the *SYSTAT* program. The tumour growth curves were analysed using a Macintosh version of the *EXPFIT* program [18] adapted to compute exponential growth curves. For each experimental group, the exponential phase of the growth curves was preliminary evaluated on the basis of the best fitting obtained, considering different sections of the curves. The growth rate was then calculated and compared, considering the part of the curves in which exponential growth was assessed. Each point on the curves corresponds to the mean tumour value from eight animals; variability of the experimental data was included in the fitting procedure as a weighting function. Since the least-square non-linear fitting procedure generates approximate standard errors, percent coefficient of variations (%CV) of the parameter R (growth rate) are reported as the estimated error of each curve. Significant differences ($P<0.05$) among the exponential growth curves of the experimental groups were detected by the F-test, performed by the *EXFIT* program, on the least-square means of the fitting before and after imposing equality constraints on the parameters (i.e. the growth rate) [18]. The Student *t*-test (two-tailed) was used to compare cytokine concentrations in the experimental groups.

3. Results

3.1. Effect of topotecan treatment on CpG-induced IL-12 and IFN-γ production

In order to define the combination protocol for the two therapies, we evaluated the suppressive activity of topotecan on the CpG-induced immune response. Production of the Th1-promoting cytokines IL-12 and IFN-γ, which are hallmarks of an immune response to CpG-ODN [19,20], was analysed in response to intravenous injection of CpG-ODN in topotecan-pretreated mice (1, 3, 7 or 14 days before) (Fig. 1). As expected, examination of splenocytes removed 2 h after CpG-ODN injection revealed high production of both cytokines. In mice previously (3 days) treated with topotecan, a slight non-significant inhibition of IL-12 production was observed, which returned to normal after 14 days

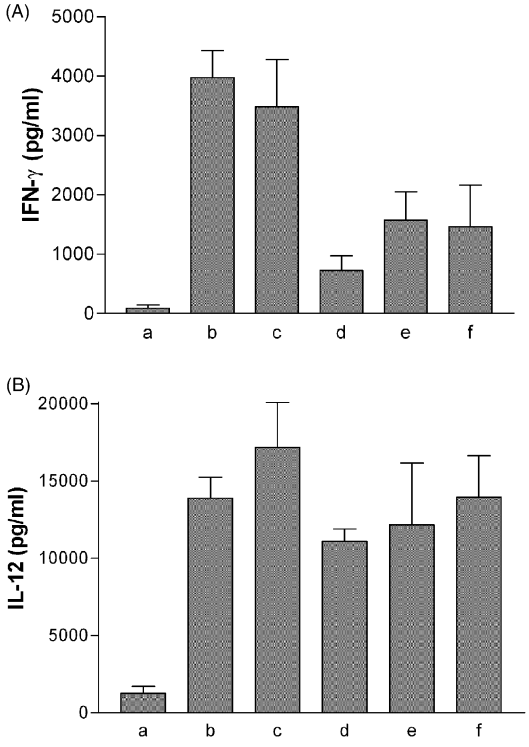

Fig. 1. Production of interferon-γ (A) and interleukin 12 (B) evaluated by enzyme-linked immunoassay in supernatants of spleen cells obtained from athymic nude mice untreated (a), treated intravenously with 200 μg/mouse CpG-ODN alone (b), or 1 day (c), 3 days (d), 7 days (e), 14 days (f) after topotecan (15 mg/kg).

from treatment, whereas IFN-γ production was significantly inhibited by topotecan treatment, with the peak of inhibition (about 85%) at 3 days after the drug treatment ($P = 0.003$ by unpaired t-test), and only a partial recovery even after 14 days.

3.2. Antitumour effects of topotecan plus CpG-ODN

In the light of the differential effect of topotecan on CpG-induced IFN-γ and IL-12 production, the antitumour effect of CpG-ODN was investigated by administering it during or after the optimal regimen of the cytotoxic drug (alternating or sequential protocol). The effect of the systemic administration of CpG-ODN in combination with a chemotherapy treatment on the growth of pre-existing tumours was evaluated in nude mice xenografted with the human PC-3 prostatic tumour, starting when tumour size averaged 100 mm^3 (Table 1; Fig. 2). In terms of growth inhibition versus control tumours (cTVI%; Table 1), mice treated with CpG-ODN alone (40 μg every third/fourth day, seven times) showed, at maximum, 50% inhibition of tumour growth, whereas mice treated with topotecan (q7d×3) showed a strong decrease in mean tumour size up to 95%, but tumour growth resumed about 3 weeks after the cessation of treatment. In all the groups of mice receiving CpG-ODN in addition to chemotherapy, cTVI% did not significantly differ from that achieved in mice treated with topotecan only (range, 95%–97%). However, in these groups a significant delay in tumour growth was observed, resulting in tTVI versus that of topotecan-treated mice ranging between 41% and 64%.

From the analysis of the curves (Fig. 2; Table 2) it appears that, in the time interval considered, control and CpG-ODN-treated tumours showed exponential growth, and that treatment with CpG-ODN induced a persistent decrease of tumour growth rate. Topotecan induced almost complete inhibition of tumour growth, but, about 20 days after its withdrawal, when tumour size started to increase, the growth rate was very similar to that observed in untreated animals. The analysis of

Table 1
Antitumour efficacy of CpG-ODN (ODN), topotecan (TPT) and TPT combined with ODN on PC3 human prostate carcinoma xenografts

Drug[a]	Treatment sequence[b]	cTVI%[c]	tTVI%[d]	Tox/Tot[e]
ODN 40	–	50	–	0/8
TPT	–	95	–	0/8
ODN 40 + TPT	Alternating	97	64	3/8
ODN 20 + TPT	Alternating	95	41	0/8
TPT + ODN 40	Sequential	96	51	0/8

[a] CpG-ODN 40 or 20 μg/mouse intraperitoneally; from day 9, q3–4d×7–10 times in the alternating treatment; from day 33, q7d×7 times in the sequential treatment. Topotecan, 15 mg/kg orally, q7d×3, at days 12, 19, 26.

[b] Treatment began when tumour volume was around 100 mm^3, starting with CpG-ODN at day 9 in the alternating sequence, or with TPT at day 12 in the sequential protocol.

[c] The optimal tumour volume inhibition percentage in treated versus control mice.

[d] The optimal tumour volume inhibition percentage in combination-treated versus TPT-treated mice.

[e] Lethal toxicity, number of toxic deaths/total number of mice in the group.

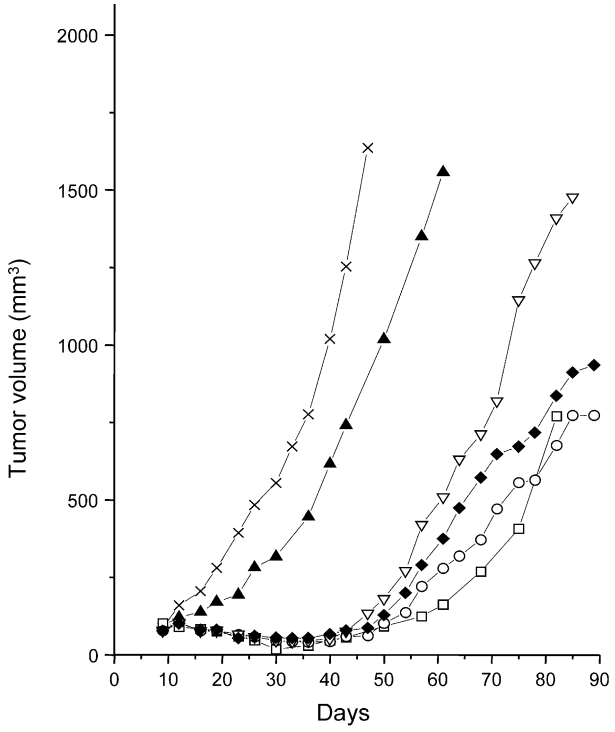

Fig. 2. Antitumour effect of chemoimmunotherapy on human PC3 prostate carcinoma xenografts. TPT was delivered orally, 15 mg/kg, in all groups. CpG-ODN (ODN) was delivered intraperitoneally, 20 or 40 μg/mouse. Each point represents the mean tumour weight from eight mice. Similar results were found in two independent experiments. Symbols are reported in Table 2.

Table 2
Effects of CpG-ODN (ODN), topotecan (TPT) and TPT combined with ODN on the exponential phase of the growth curves of PC3 human prostate carcinoma xenografts, analysed by the *EXPFIT* program

Drug[a]	Growth rate[b] (mg/day)	Doubling time[b] (days)	CV[c] (%)
× Solvent	0.061	11.3	0.4
▲ ODN 40	0.048	14.4	0.5
▽ TPT	0.059	11.7	1.2
□ ODN 40 + TPT	0.048*	14.4	2.3
◆ ODN 20 + TPT	0.054*	12.8	4.4
○ TPT + ODN 40	0.042*	16.5	3.0

*$P < 0.01$ versus TPT-treated tumours.

[a] See Table 1 footnote 'a' for details.

[b] The growth rate corresponds to the parameter R of the *EXPFIT* analysis [18]; the doubling time was calculated as $0.6931/R$.

[c] CV, coefficient of variation of the parameter R.

the exponential phase of the topotecan-treated group versus that treated with topotecan + CpG-ODN showed that the combined treatment induced a significant decrease in tumour growth rate ($P < 0.01$, for all three groups). Indeed, topotecan at the optimal dose and schedule delayed the time at which mean tumour volume reached 1 cm^3 by about 35 days, and the addition of CpG-ODN prolonged that time by at least another 20 days.

Body weight loss during treatments never exceeded 10%; no lethal toxicity was observed in any group receiving the different protocols, except in the group treated with CpG-ODN (40 μg/mouse) + topotecan in the alternating schedule, where lethal toxicity was evident and three out of eight mice died during the treatment time, at days 16 (one) and 20 (two), that is after one or two topotecan treatments (Table 1).

In order to investigate whether increased toxicity was related to the specific experimental conditions (athymic tumour-bearing mice, treatment with topotecan), other toxicity experiments were performed using euthymic non-tumour-bearing mice and another cytotoxic drug, doxorubicin (a topoisomerase II inhibitor). As shown in Fig. 3, the alternating treatment of doxorubicin (7.5 mg/kg, intraperitoneal, q7d×3) and CpG-ODN (40 μg/mouse, intraperitoneal, q7d×3) induced lethal toxicity, with five of nine mice dead by day 21. No toxicity was observed in mice treated with each single agent, except for one of 30 mice in the doxorubicin-treated group. Consistent with the results in experiments performed with topotecan, toxicity was not observed when CpG-ODN (40 μg/mouse) was given, starting 1 week after the last doxorubicin treatment (not shown).

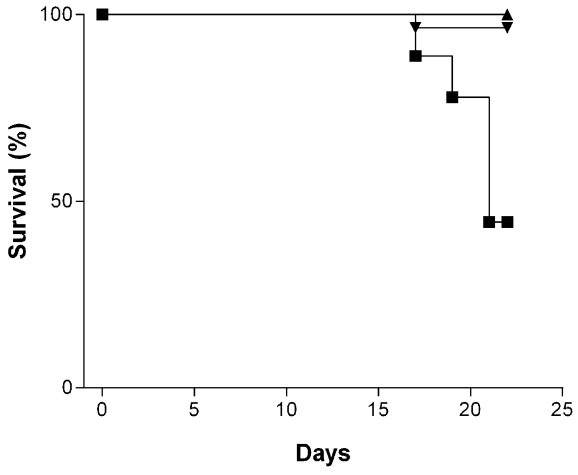

Fig. 3. Surviving percentage of healthy euthymic FVB mice treated with: (▼) doxorubicin 7.5 mg/kg, intraperitoneally, q7d×3, at days 4, 11, 18 (30 mice); (▲) CpG-ODN, 40 μg/mouse, intraperitoneally, q7d×3, at days 1, 8, 15 (10 mice); (■) doxorubicin alternated with CpG-ODN (9 mice).

3.3. Effect of topotecan treatment on CpG-induced TNF-α production

Macrophages reportedly produce large amounts of TNF-α after CpG-ODN stimulation [21,22]. Topotecan acts as a cell sensitiser to death receptor-induced apoptosis [23], which can be activated by different ligands of the TNF family (FasL, TRAIL, TNF) [24]. Thus, variations in TNF concentrations may critically affect pathways of cell death [25]. Analysis of the serum TNF-α in topotecan-pretreated mice at 2 h after the intravenous injection of CpG-ODN, when the serum TNF-α reportedly peaks [26], revealed a drastic reduction not an increase (Fig. 4).

4. Discussion

The present results demonstrate that, in mice xenografted with human PC-3 prostate carcinoma, the combination of a topoisomerase I inhibitor and CpG-ODN retards tumour growth in comparison to the effect of chemotherapy alone. To reproduce better the clinical condition, CpG-ODN was delivered by a systemic route. Indeed, it is well established that peritumoral treatment of mice with CpG-ODN can activate the innate immune response to a tumoricidal state and lead to a decrease in tumour growth rate [6,7,10], but such an approach is often unsuitable in patients. In our study, the significant delay in tumour growth achieved with all the combination protocols indicated strong and reproducible therapeutic benefits of systemically administered topotecan + CpG-ODN, despite the immune suppression induced by the drug treatment. When repeated administrations of the immonostimulator were begun after the end of the full chemotherapy regimen, tumour control was maintained for a long time without any sign of toxicity. The retardation of tumour growth is of

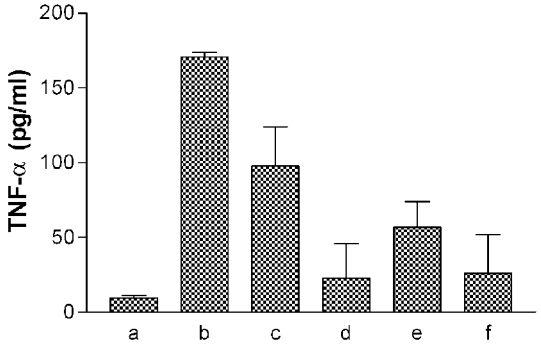

Fig. 4. Tumour necrosis factor-α serum concentration evaluated by enzyme-linked immunoassay in athymic nude mice. Untreated (a), treated intravenously with 200 μg/mouse CpG-ODN alone (b), or 1 day (c), 3 days (d), 7 days (e), 14 days (f) after topotecan (15 mg/kg).

particular relevance considering that topotecan, in its optimal therapeutic regimen, was able to achieve a rather complete growth inhibition of the PC-3 tumour. It is also possible that the strong efficacy of topotecan might have partially clouded the contribution of the immunomodulator in the antitumour activity of the combination. Indeed, another study on PC-3 tumour xenografts combining a less effective chemotherapy and a different immunostimulating agent reports more favourable results of the combination versus the cytotoxic drug itself [27].

Cytokine production, instead of immune cell count, served as an indicator of the suppressive activity of topotecan on the CpG-induced immune response. This factor is a better indicator of the immune cell response than is the cell count, since after chemotherapy some cells are still alive but no longer able to respond to immune stimulation. While ODN-induced IFN-γ production was significantly inhibited, IL-12 was only slightly reduced by the chemotherapy, indicating that topotecan has a minimal effect on the activity of IL-12-producing cells, such as dendritic cells and macrophages, activated by CpG-ODN. The induction of IL-12 by CpG-ODN could be responsible of the increased antitumour effects achieved by the combined protocols.

A major finding concerned the lethal toxicity of the chemo-immuno combination. Indeed, 37% mice died when topotecan + CpG-ODN (40 μg/mouse) was used in the alternating protocol. No evidence of toxicity was observed when the same doses of drugs were used in a sequential protocol, suggesting a critical role for the sequence. Moreover, lethal toxicity was observed in healthy mice when a different drug, the topoisomerase II inhibitor doxorubicin, was combined at its maximum tolerated dose with CpG-ODN in an alternating sequence; 55% of mice died of toxicity during the treatment period when the immunomodulator (40 μg/mouse) was delivered by the alternate and not by the sequential schedule. No toxicity was reported in a recent study combining topotecan + CpG-ODN at a very high dose (100 μg/mouse), but only a single chemotherapy treatment followed by repeated CpG-ODN was investigated [28].

The strong toxicity observed when CpG-ODN was alternated with a chemotherapeutic drug was somewhat unexpected, since various preclinical and clinical studies have never reported toxicity from these immunomodulators [2]. Moreover, the use of a bacterial synthetic lipopeptide in combination with the cytotoxic drug irinotecan allowed a higher dose of the drug to be delivered in nude mice, due to the protective effect of the oral immunomodulator against the drug-induced gastrointestinal toxicity [29]. As our results could not be ascribed to TNF-α-related toxicity (because the serum TNF-α level was decreased in our combination protocols), the interaction of toxicity at the gastrointestinal level was considered. However, histological analysis of samples of small intestine from mice after treatment with the alternating protocol revealed no increase in intestinal damage (not shown). The early death of mice (during the treatment period) might instead be related to myelosuppression, i.e. CpG-ODN, by stimulating extramedullary haematopoiesis and/or mobilisation of stem cells, might increase chemotherapy-induced myelotoxicity. Indeed, CpG-ODN reportedly stimulates extramedullary murine haematopoiesis, with increased frequencies of stem cells in the spleen (i.e., B220/CD3 double-negative cells) [30], and it induces the release of IL-8 from neutrophils, macrophages and dendritic cells *in vitro*, which is known mobilising stem cells from the bone marrow [20,31–33]. Accordingly, preliminary results indicate significant concentrations of IL-8 in the serum of mice 2–4 h after CpG-ODN treatment. Moreover, the appearance of stem cells in the blood (revealed as colony-forming units) a few days after CpG-ODN treatment (unpublished results) supports the hypothesis that myelosuppression is a relevant mechanism for the combined toxicity. If myelosuppression is the primary toxic mechanism, CpG-ODN might be more suitably combined with cytotoxic drugs that produce gastrointestinal damage (e.g. irinotecan) rather than myelosuppression (e.g. topotecan and doxorubicin) as dose-limiting toxicity.

In conclusion, our study on a human prostate carcinoma xenograft indicates that the combination of a very effective cytotoxic drug and an immunostimulating agent enhances the antitumour response, mainly by retarding tumour growth. The combination produced unexpected toxicity when the two therapies were administered alternately, suggesting that the sequential schedule (optimal chemotherapeutic regimen followed by repeated immunostimulatory challenges) is the most appropriate for further investigation.

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