Neuroblastoma is one of the most common extracranial childhood solid tumors (1). Neuroblastoma arises from the sympathetic nervous system and is frequently diagnosed when already metastatically widespread to sites other than its original localization (most frequently the medullary adrenal gland). Metastases can involve several organs, with bone marrow representing the most frequent site (1). In spite of the recent treatment protocols involving intensive chemotherapy, surgery, external beam, and/or metabolic radiotherapy and hematopoietic stem cell transplant, the long-term prognosis of the disease in its advanced stages remains poor (2). Moreover, the general and organ-specific toxicity of the treatment regimens poses serious limitations to the doses of drugs that can be safely given, thus reducing the chance to kill the totality of tumor cells before they develop multidrug resistance. Therefore, although in many cases an apparent complete or partial remission can be achieved, recurrence of disease within few months to few years is quite common (3). This is likely due to the persistence of circulating neuroblastoma cells that can be detected by various techniques in the bloodstream of patients, and their presence is a poor prognostic factor for the outcome of the disease (4).

When relapse takes places, tumor cells are often pluri-resistant even towards drugs that were not included in first-line treatment, and disease progression becomes overwhelming. Given this situation, two major aims in neuroblastoma therapy should be pursued: first, to achieve an as most thorough as possible (ideally total) tumor cell kill during first-line treatment; second, trying to eliminate circulating neuroblastoma cells and to localize and sterilize the often tiny sites of metastatic foci that residue after induction therapy before they give rise to relapse.

One of the most interesting pharmacologic strategies recently developed involves passive or active selective drug targeting to tumor cells, with relative spare of healthy cells and tissues. This can be achieved by different means, most of them involving the “packaging” of antitumor drugs at high concentrations within submicrometer particles of different chemical composition (liposomes and different synthetic polymers; refs. 5–9). Once injected systemically, these vehicles remain into circulation, shielding normal organs from the toxic effects of the drugs they contain, unless they reach anatomic regions were the endothelial walls of blood vessels are damaged, with ample discontinuities between endothelial cells, allowing them to extravasate.
to the extracellular environment (5–9). This situation is typical (although not specific) of both primary and metastatic tumor sites, where the vasculature is constantly undergoing structural changes to meet the increase in oxygen supply required by growing tumor tissue (6, 7, 10). To our knowledge, however, none of the compounds employed to build up these nanoparticles displayed an antitumor activity by itself, independent of the encapsulated drug. To this purpose, we have developed a new synthetic amphiphilic polymer constituted by a polyvinyl alcohol backbone substituted at a low (~1.5%) molar ratio with both a hydrophobic (oleyl alcohol) and a hydrophilic (tetraethylene glycol monoethyl ether) moieties. This polymer will be referred to as P10(4) because of the average 10-kDa molecular weight of the polyvinyl alcohol backbone. Due to its amphiphilic nature, P10(4), when dissolved in aqueous medium, spontaneously self-assembles to give rise to nanospheres of average diameter of 150 to 200 nm. Through techniques whose details are beyond the scope of this article and that have been described elsewhere (11), nanospheres of P10(4) can provide an excellent and simple, low-cost vehicle for mildly hydrophobic drugs (as is the case for several cytotoxic compounds).

Before even an experiment with P10(4)-drug conjugation, we wanted to evaluate the potential toxicity of the polymer on both normal and tumor cells in vitro. To our surprise, P10(4) turned out to exert by itself a powerful cytotoxic activity on both neuroblastoma and melanoma cell lines, while totally lacking effect on normal resting peripheral blood lymphocytes and only modestly affecting the viability of cycling, stimulated lymphoblasts and normal human fibroblasts. Although we are still investigating the primary triggering event, we showed that cytotoxicity takes place through induction of programmed cell death.

In vivo experiments with two models of pseudometastatic neuroblastoma in nude mice also confirmed a significant antitumor activity of P10(4) alone.

**Materials and Methods**

**Cell lines, culture conditions, and isolation of peripheral blood mononuclear cells.** To broadly cover the phenotypes exhibited by neuroblastoma and melanoma cell in vitro, we used three human neuroblastoma cell lines (SH-SY-5Y, HTLA-230, IMR-32), these two latter being MYCN amplified), one murine neuroblastoma cell line (NXXS2), and two human melanoma cell lines (M2Z-MEL and RPMI 7932; refs. 12–14). Cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with L-glutamine, penicillin/streptomycin (Sigma), and 10% fetal bovine serum (Corning, Inc., Corning, NY) in the presence or absence of P10(4) at a concentration of 1, 2.5, and 5 μg/mL for 24 hours. Viable cells were counted with trypan blue dye (Sigma), resuspended in complete medium containing 0.3% Agar Noble (Difco Laboratories, Detroit, MI), and seeded at a concentration of 1,000 viable cells/mL in 0.6% Agar Noble layers. Colony formation was assessed by microscopic inspection and counting 15 days after cell seeding.

**Endotoxin testing on P10(4) preparations.** To exclude that any cytotoxic effect could be due to endotoxin contamination, we did endotoxin assay on different batches of P10(4) using the Limulus Amebocyte Lysate, Pyrotest (Associates of Cape Cod, Inc., Falmouth, MA), according to the manufacturers instructions.

**DNA fragmentation assay.** After various treatments, the cells were washed with ice-cold PBS, centrifuged at 1,200 rpm for 10 minutes, and processed following the method described by Pagnan et al. (15). Samples were analyzed on 1.2% agarose gel electrophoresis, in 40 mmol/L Tris acetate, and 1 mmol/L EDTA (Sigma; TAE) buffer (pH 8). One microgram of 1 kb Plus DNA Ladder (Invitrogen, San Giuliano Milanese, Italy) was applied to each gel to provide molecular size markers.

**Caspase activity assay.** Detection of caspases activity was evaluated by ApoFluor Green Apoptosis Detection Kit specific for caspase-1 and caspase-4, caspase-2, caspase-3 and caspase-7, caspase-6, caspase-8, caspase-9, caspase-10, and caspase-13 (MP Biotechnologies, Verona, Italy), according to the manufacturer’s instructions. Briefly, cells were detached with EDTA and centrifuged at 400 × g for 5 minutes at room temperature. Cell supernatants were removed, and the pellets were resuspended in a buffer containing the appropriate caspase-specific fluorescent probe. After 1 hour of incubation, samples were washed and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) equipped with a 15-mW argon ion laser at 488 nm (16).

**Pan-caspase inhibition test.** To evaluate the role of caspase activation in the cell death induced by P10(4), the cell lines were preincubated for 1 hour with the general caspase inhibitor z-VAD-fmk (Sigma; 50 μmol/L) and then treated with vehicle or P10(4) (2.5 and 5 μg/mL for neuroblastoma and melanoma cells). The effects of caspase activity inhibition on cell viability were evaluated by flow cytometry after 6 and 24 hours of incubation for neuroblastoma lines and 24 hours and 48 hours of incubation for melanoma lines.

**Colony formation by soft agar assay.** SH-SY-5Y, IMR-32, HTLA-230, and NXS2 neuroblastoma cells were plated into six-well plates (Corning, Inc., Corning, NY) in the presence or absence of P10(4) at a concentration of 1, 2.5, and 5 μg/mL for 24 hours. Viable cells were counted with trypan blue dye (Sigma), resuspended in complete medium containing 0.3% Agar Noble (Difco Laboratories, Detroit, MI), and seeded at a concentration of 1,000 viable cells/mL in 0.6% Agar Noble layers. Colony formation was assessed by microscopic inspection and counting 15 days after cell seeding.

**Binding of P10(4) to neuroblastoma cell lines.** P10(4) molecule was radioiodinated according to the chloramine method described by Tabata et al. (17). SH-SYSY neuroblastoma cells (200,000 per well) were treated with 125I-labeled P10(4) at the final concentration of 2.5 μg/mL (160,000 counts per minute/microwell) for 5 minutes, 1, 2, and 4 hours at 37°C and 4°C. Then, the cells were detached with EDTA solution, washed twice with PBS, and counted in a Packard Cobra II auto gamma counter.

**Pharmacokinetic experiments.** Four- to 5-week-old female CD1 nude/male mice, weighing 18 to 25 g, were purchased from Harlan Laboratories (Udine, Italy) and housed in sterile enclosures under specific virus and antigen-free conditions. All procedures involving mice and their care were in accordance with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/109, OIL 358, Dec. 1, 1987 and NIH Guide for the Care and Use of Laboratory Animals). CD1 nude/male mice in the weight range of 20 to 30 g were injected via the tail vein (i.v.) with a single dose of 125I-labeled P10(4) (70 μg/200,000 counts per minute/mouse). At selected time points (5, 15, and 30 minutes and 1, 2, 6, 12, and 24 hours) postinjection, mice (3 mice per group) were anesthetized with halothane and sacrificed by cervical dislocation. A blood sample (100 μL) was collected by heart puncture and counted for the 125I label in a Beckmann 8000 gamma counter. Blood correction factors were applied to all the sample (7). Lung, kidneys, thyroid, liver, spleen, and heart were removed; weighed; and counted for the 125I label. Pharmacokinetic variables were calculated using the software package PKAnalyst, version 1.1 (MicroMath Scientific Software, Salt Lake City, UT).

**In vitro therapeutic experiments.** For therapeutic experiments, CD1 nude/male mice were treated i.v. with HTLA-230 (5 × 106 per mouse; 12 mice per group) and NXS2 (100,000 per mouse; 9 mice per group) as described (18).
Twenty-four hours after the tumor cell inoculation, the animals were treated with P10(4) (300 mg/kg) or vehicle alone (PBS), given slowly through the tail vein in a volume of 200 μL. The treatment was repeated after 7 days. To determine treatment efficacy, the animals were monitored routinely for weight loss and general behavior, and survival time was used as the main criterion. The statistical significance of differential survival between experimental groups of mice was determined by Kaplan-Meier curves and log-rank (Peto) test by the use of StatDirect statistical software (CamCode, Ashwell, United Kingdom).

Results

Schematic chemical structure of P10(4). A schematic model of the chemical architectural structure of P10(4) is illustrated in Fig. 1.

P10(4) induces apoptosis on neuroblastoma and melanoma cell lines. The effects of P10(4) on neuroblastoma cells were evaluated on cultures of three human (SH-SY-5Y, HTLA230, and IMR32) and a murine (NXS2) neuroblastoma cell lines, whereas for melanoma, two human cell lines were used (i.e., MZ2-MEL and RPMI 7932). As a normal, nonmalignant counterpart, resting or cycling PBMC and fibroblasts were used.

The dose-response curves for neuroblastoma and melanoma cell lines and normal cells are shown in Fig. 2. Time course and dose response experiments showed a time-dependent and dose-dependent progressive loss of cell viability, characterized by initial cell shrinking and rounding, soon followed by cell detachment from the flask. At higher magnification, nuclear condensation and shrinking could be observed (data not shown). Detached cells maintained their morphologic integrity for a relatively long period after loosing contact (up to 3 days) which, together with the above optical microscope observations, suggests that cell death takes place through apoptosis rather than necrosis. This was also confirmed by agarose gel electrophoresis of DNA, where the internucleosomal fragmentation of DNA typical of programmed cell death could be observed in both neuroblastoma and melanoma cell lines (Fig. 3A) The IC50 of P10(4) on the various neuroblastoma and melanoma cell lines after 24 and 48 hours of treatment are shown in Table 1. This table also shows the modest toxicity of P10(4), towards cycling and the virtual lack of effect on resting PBMC and fibroblasts. Cytofluorimetric analysis of DNA content showed that in both neuroblastoma and melanoma cell lines, there was a dose-dependent and time-dependent increase of cells in the sub-G1 phase of the cell cycle upon treatment with P10(4), again suggestive for apoptotic cell death (Fig. 3B).

Effects of P10(4) on clonogenic potential of neuroblastoma cells. To investigate whether P10(4) affected the ability of neuroblastoma cells to form colonies in soft agar, cells were treated for 24 hours with different doses of P10(4). The same number of viable cells (as judged by trypan blue exclusion assay) from control and treated cultures were then seeded at low cell density in agar. After 15 days, the deriving colonies were visualized and counted microscopically. Figure 4 shows the pattern of dose-response inhibition of colony formation by neuroblastoma cells. The number of colonies and their size were strongly diminished in treated cultures, and, at the higher doses, the growth of colonies was totally inhibited.

Involvement of caspase activation in P10(4)-induced cell death. To further confirm the apoptotic nature of cell death induced by P10(4) in tumor cells, and in an attempt to clarify the underlying primary mechanisms involved in this process, we first evaluated whether caspases were involved. To this aim, we first used a fluorescence-based pan-caspase activity assay kit. The results showed a dose-dependent significant shift in fluorescence in both neuroblastoma [SH-SY-5Y, 73% fluorescence increase at 24 hours after treatment with 5 μg/mL P10(4)] and melanoma cells [RPMI 7930, 88% fluorescence increase at 24 hours after treatment with 10 μg/mL P10(4); data not shown]. When cells were preincubated with the general caspase inhibitor z-VAD-fmk before treatment with P10(4), caspase activation was blocked as was the cellular apoptosis (Fig. 3B). We then did the same analysis using all the commercially available kits for single caspases (i.e., caspase-1, caspase-2, caspase-3, caspase-6, caspase-8, caspase-9, caspase-10, and caspase-13) and measured the increase in fluorescence after 1, 2, 4, 6, 12, and 24 hours. As shown in Fig. 5, all the
above caspases were activated already at 4 hours and became more active thereafter in treated neuroblastoma cells with respect to controls. Although these experiments did not allow us to individuate which caspase(s) was activated first, they clearly confirmed the apoptotic nature of cell death induced by P10(4).

Lack of binding of P10(4) to neuroblastoma and melanoma cells. To understand the nature of the physical interaction between cells and P10(4), we labeled P10(4) with 125I and evaluated the amount of cell-associated radioactivity after 5 to 120 minutes of incubation at both 4°C and 37°C. In our conditions, we could never detect any significant cell-associated radioactivity. Moreover, upon gel filtration chromatography (Sephadex G50) of the supernatants of these cultures, the totality of the radioactivity was recovered in the breakthrough fraction of the column, showing that P10(4) had maintained its nanospheric structure, and that no metabolic processing implying breakdown and loss of the radioactive moiety took place. Thus, the nature of cell-P10(4) interaction remains obscure.

**Pharmacokinetics and biodistribution of P10(4) in vivo.** To evaluate the pharmacokinetic properties of P10(4) in vivo, 125I-labeled P10(4) (~100 nCi/mouse) was used as a tracer and mixed with unlabeled P10(4). Three mice for each time point were injected i.v. with a single dose of 70 µg P10(4). At the desired times, mice were sacrificed, and the radioactivity was measured by γ-counting blood and the major organs. Figure 6A describes the curve of blood concentration of P10(4) over time. As expected, due to the particulate nature of P10(4), a long elimination half-life of ~9.5 hours was observed. The main pharmacokinetic variables are shown in Table 2. The distribution of P10(4) in the examined organs is shown in Fig. 6B. As can be seen, a high percentage of injected P10(4) remains in the bloodstream, with very little accumulation in the major organs.
Table 1. EC50 of P10(4) on neuroblastoma and melanoma cell lines and on normal cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EC50 (nmol/L)</th>
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<tbody>
<tr>
<td>Neuroblastoma</td>
<td>24 h</td>
</tr>
<tr>
<td>SH-SY-5Y (human)</td>
<td>480 ± 120</td>
</tr>
<tr>
<td>IMR-32 (human)</td>
<td>230 ± 70</td>
</tr>
<tr>
<td>HTL2A-230 (human)</td>
<td>790 ± 90</td>
</tr>
<tr>
<td>NXS2 (murine)</td>
<td>340 ± 80</td>
</tr>
<tr>
<td>Melanoma</td>
<td>48 h</td>
</tr>
<tr>
<td>M22-MEL (human)</td>
<td>570 ± 70</td>
</tr>
<tr>
<td>RPMI 7932 (human)</td>
<td>730 ± 90</td>
</tr>
<tr>
<td>Normal cells (human)</td>
<td>40 h</td>
</tr>
<tr>
<td>Resting PBMC</td>
<td>&gt;6,000</td>
</tr>
<tr>
<td>Cycling PBMC</td>
<td>&gt;6,000</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>&gt;6,000</td>
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Noteworthy, the relatively low thyroid-associated radioactivity suggests that P10(4) does not undergo extensive catabolism (or, at least, deiodination) in vivo.

In vivo antitumor activity of P10(4). To mimic the clinical features of metastatic neuroblastoma, we injected CD1 nude/mice with a human (HTL2A-230, 5 x 10^6 cells per mouse, 12 mice per group) or a syngeneic murine (NXS2, 1 x 10^6 cells per mouse, 9 mice per group) i.v. The mice were then randomly assigned to the control or treatment groups. Treated mice received two injection of P10(4) (300 mg/kg, i.v., in sterile saline solution) on days 1 and 7 after tumor cell injection, whereas control mice received the same volume (200 μL) of vehicle. Three times a week, mice were monitored for their body weight, general physical, and performance status, as well as for externally visible tumor mass or ascites formation. Animals were sacrificed whenever excessive (>25%) weight loss, huge tumor growth, massive ascites, or impairment of motor functions due to spinal cord compression took place. Figure 7 shows the survival profile of treated versus control mice engrafted with NXS2 (A) or HTL2A230 (B). In both cases, a highly significant (P < 0.001, log-rank test Peto) increase in mean survival time could be observed in mice that received P10(4). Moreover, 3 of 9 mice (33%) engrafted with NXS2 and 3 of 12 mice (25%) engrafted with HTL2A-230 were healthy and alive at the end of the experiment (120 days after tumor inoculum) and showed no evidence of macroscopic disease at necropsy. In addition, whereas control mice underwent rapid and extensive metastatic tumor growth, involving mainly adrenal gland, kidney, ovary, liver, spleen, and bone marrow, in treated mice, these events took place more slowly and in a less extensive amount. Overall, even in treated mice that eventually died, the course of the disease was less overwhelming.

Discussion

The concept of encapsulating antitumor drugs into submicrometric particles to protect healthy tissues and, possibly, target their cytotoxic effects to primary and metastatic lesions is a relatively recent development in cancer pharmacology (6, 8). Moreover, this strategy also contributes to improve the solubility in biological fluids of several mildly to highly lipophilic drugs (5). Several approaches have been undertaken with different chemical composition of these drug vehicles, with liposomes, and their actively targeting form (immunoliposomes), being perhaps the most suitable (12, 18). An extensive review of the several approaches to nanoparticle-mediated drug targeting is provided by Moghimi et al. (9). We recently developed a synthetic polymer, referred to as P10(4) in which a polyvinyl alcohol–based backbone is rendered amphiphilic through a low degree of substitution with both lipophilic (oleyl alcohol, 1.5%) and hydrophilic (tetraethylene glycol monoethyl ether, 1%) moieties. The synthetic procedures and the chemico-physical properties of the polymer and the deriving nanospheres are the subject of a separate article (11). Before even trying to encapsulate drugs into these nanospheres, we wanted to ascertain the possible cytotoxicity of our compound on both normal (resting or cycling human PBMC and fibroblasts) and tumor cells (neuroblastoma and melanoma cell lines) in vitro. P10(4), in the range of 2.5 to 50 μg/mL, turned out to be strongly cytotoxic only to tumor cells but not on PBMC and fibroblasts, as assessed by trypan blue exclusion assay. To clarify if P10(4)-mediated toxicity took place only on cycling cells, we collected PBMC and stimulated them with the mitogen phytohemagglutinin before treating these cells with the same doses of P10(4) used in the above experiments; we could observe a certain degree of cell death only at higher doses and longer times, the toxicity being anyway much lower than that observed on tumor cell lines. This finding, along with the broad differences of IC50 of P10(4) on the various cell lines, suggests a specific mechanism of action rather than a generic toxic effect. We then proceeded to clarify whether P10(4)-induced cell death took place through necrosis or apoptosis. The simple microscopic inspection of treated cultures suggested this latter hypothesis: the earliest event was a dose-dependent and time-dependent phase, during which the cells showed shrinking and membrane blebbing followed by cell detachment from the culture plate. At higher magnification, the nuclei began to shrink and condensate, without loss of cell integrity.
that took place only after up to 3 days of culture. Through fluorocytometry after propidium iodide staining of the cells and DNA internucleosomal fragmentation, we further confirmed that P10(4)-induced cytotoxicity was triggered by an apoptotic pathway. We next investigated the involvement of caspases activity in this process. By Western blot, we could initially document a time-dependent cleavage of caspase-3 (data not shown); preincubation of the cells with the pan-caspase inhibitor z-VAD-fmk blocked both apoptosis and caspase activation. This prompted us to further clarify the temporal hierarchy of caspase activation, using all the commercially available kits for fluorescence-based detection of caspase activity. Through a time course experiment on SH-SY-5Y neuroblastoma cells, we observed that all the caspases became activated already 4 hours after P10(4) treatment, and their activity increased with time, whereas in control cultures, the caspases' activity remained at its basal level. However, these experiments did not provide a hint on which caspase became activated first, giving rise to the cascade triggering of the other caspases. Curiously, we observed activation even of caspase-8, which reportedly is not expressed by the majority of neuroblastoma cell lines (including SH-SY-5Y) and tumors, due to
hypermethylation of its gene’s regulatory sequences (19, 20). Studies on the state of methylation of these sequences after treatment with P10(4) are currently ongoing. Should demethylation turn out to be induced by P10(4), then the interesting hypothesis to cotreat cells with both P10(4) and tumor necrosis factor–related apoptosis-inducing ligand to get a synergistic effect would raise.

The degree of sensitivity of neuroblastoma cells to P10(4) does not seem to be significantly influenced by MYCN gene amplification status, because both non–MYCN nonamplified (SH-SY5Y) and MYCN amplified (IMR-32 and HTLA-230; ref. 21) were similarly affected by P10(4) treatment. To clarify the nature of the interaction between P10(4) and tumor cells, we labeled the polymer with $^{125}$I and did time course binding experiments at both 4°C and 37°C. We could not observe any significant cell-associated radioactivity in none of the conditions and time points, suggesting that P10(4) is neither bound nor taken up by the cells. This in turn suggests that P10(4)-mediated effects on cells are due to a very transient and reversible contact with cell membrane, to which an irreversible loss of cell viability and proliferative potential ensues. This latter effect has been clearly evidenced when colony-forming assays were done. Even when apparently viable cells (i.e., still able to expel trypan blue) were plated on soft agar after a short treatment with P10(4), their ability to form colonies was dramatically reduced or completely abolished.

Due to the lack of significant binding of P10(4) to cells and the morphologic appearance of treated cells, P10(4) seems to induce apoptosis of tumor cells by interfering with their cell-cell and/or cell-matrix adhesion. Cells that normally require intercellular and cell-extracellular matrix contact for their survival and growth undergo apoptosis when these contacts are prevented (22, 23). This particular form of apoptosis is known as “anoikis” (24). The biochemical pathways signaling

![Blood clearance kinetics and biodistribution of P10(4) in CD1 nude/nude mice. A, $^{125}$I-labeled P10(4) was injected i.v. in a single bolus dose (70 μg/mouse). At different times post-injection, blood was collected and counted in a gamma-counter for $^{125}$I label. Points, average of three mice; bars, confidence interval. B, at different times post-injection, lung, kidneys, thyroid, liver, spleen, and heart were removed, weighted, and counted for the $^{125}$I label in a gamma-counter. Columns, average of three mice; bars, confidence interval.](image-url)
adhesion-mediated cell survival are very complex, involving several tyrosine and non-tyrosine kinases, with phosphatidylinositol 3-kinase/Akt being the key enzymatic complex apparently playing an essential role (24, 25). We studied whether P10(4)-treated cells exhibited lower levels of phosphorylated Akt by Western blot but could not document any inhibition of this pathway by P10(4). Moreover, we investigated whether P10(4) could down-regulate the expression of N-CAM or $\beta_1$ and $\beta_3$ integrin chains expression by cytofluorimetric assay with specific antibodies, but again, we could not detect any significant decrease in membrane expression of these adhesion molecules in P10(4)-treated cells with respect to controls. Thus, the mechanism of action of our polymer in inducing apoptosis remains largely elusive.

The experiments done in vivo in nude mice confirmed the antitumor activity of P10(4). In mice inoculated with both murine (NXS2) and human (HTLA-230) neuroblastoma cells, treatment with 300 mg/kg of P10(4), given on days 1 and 7 after tumor cells inoculum, resulted in a highly significant increase in life span. Moreover, 30% (NXS2) and 25% (HTLA-230) of mice were alive and apparently disease-free 4 months after tumor challenge, and treated animals who died or were sacrificed showed a much milder tumor burden and invasion than did control mice. Thus, the antitumor activity of P10(4) observed in vitro was confirmed in vivo. We are presently devising different treatment dosage and schedule to find out whether different regimens could further enhance the therapeutic potential of P10(4). It is interesting to note that preliminary experiments, in which mice were treated only once 24 hours after tumor cell inoculum with the same dose of P10(4) (300 mg/kg), gave rise to results in terms of mean survival time and long-term survival that, although statistically less significant, are similar to those achieved with the two treatments schedule described above. This finding suggests that...
the main effect of P10(4) derives from extensive killing of neuroblastoma cells that are circulating in the bloodstream or that are still in close proximity to endothelial cells after extravasation, thus explaining the reduced number and size of metastases observed in treated mice. Thus, P10(4) could be employed immediately after (or even during) the front-line therapy to delay or even prevent relapse generated by circulating neuroblastoma cells. Moreover, due to the lack of toxicity of P10(4), this compound could be given for long periods of time.

Pharmacokinetic and biodistribution analyses showed a prolonged blood circulation half-life (~9.5 hours) as it would be expected based on the somanovl architectural organization of P10(4), giving rise to a high area under the curve value. No particular organ accumulation of P10(4) could be observed, excepted a slight concentration in kidneys, likely the main organs of elimination of the polymer. This long circulation half-life and elevated area under the curve should provide a prolonged exposure of tumor cells to the proapoptotic effects of P10(4). As long as a potential toxicity is concerned, we did not observe any acute untoward effect with dosage level twice as high that used in therapeutic experiments. Polyvinyl alcohol by itself is biocompatible (26), and the small amounts of oleyl alcohol and tetraethylene glycol monoethyl ether are unlikely to exert toxic effects at the dosage that proved to be therapeutic.

Another interesting feature of the potential antitumor effect of P10(4) is that this molecule, unlike the great majority of current drugs used in cancer treatment, does not seem to interact with cellular DNA. This property would circumvent the occurrence of drug resistance, which is one of the main challenges clinicians must withstand and counteract, and that is the most frequent cause of treatment failure. Taken together, our findings suggest that P10(4) holds promise as an effective antitumor compound by itself, independent of its possible function as a carrier for other more established drugs. This latter possibility is presently being investigated with fenretinide (4-HPR), a synthetic retinoid with well-established proapoptotic activity but whose parenteral administration is strongly hindered by its poor water solubility (14, 15, 27). The mechanism of action of P10(4) at the cellular and molecular level is presently a further focus of our investigations, with the hope to develop other, possibly more effective, polymer-based moieties for cancer treatment.

References

In vitro and In vivo Antitumor Activity of the Novel Derivatized Polyvinyl Alcohol-Based Polymer P10(4)

Lizzie Raffaghello, Guendalina Zuccari, Roberta Carosio, et al.


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