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# Taxane-Antibody Conjugates Afford Potent Cytotoxicity, Enhanced Solubility, and Tumor Target Selectivity[1]

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## ABSTRACT

**Paclitaxel (Taxol) is a chemotherapeutic agent that prevents disassembly of microtubular polymers, causing a growth arrest in the $G_2$-M phase of the cell cycle and leading to apoptotic death. Paclitaxel has remarkable efficacy against fast-growing tumors but possesses major drawbacks, such as poor solubility and lack of tumor selectivity. Conversely, monoclonal antibodies usually have low therapeutic efficacy but are highly soluble and selectively target tumor markers overexpressed in cancer cells. Therefore, to improve the therapeutic index of taxanes as chemotherapeutics, the high toxicity of paclitaxel was combined with the high selectivity and solubility of monoclonal antibodies as targeting agents. We report the chemical coupling and characterization of paclitaxel-antibody conjugates for treatment of neuroectoderm-derived tumors. Paclitaxel-antibody conjugates afforded selective toxicity toward cells expressing the target marker and were more cytotoxic *in vitro* than equimolar concentrations of free paclitaxel or free paclitaxel plus free antibody. In an *in vivo* model of xenografted tumors, systemic administration of paclitaxel-antibody conjugates prevented tumor growth and prolonged survival of mice better than free drugs. In addition, paclitaxel-antibody conjugates were highly soluble in water and stable at −20°C for at least 3 months. These studies may lead to an increase or an improvement of the armamentarium and selectivity of cytotoxic agents.**

## INTRODUCTION

The clinical use of chemotherapeutic agents against malignant tumors is successful in many cases but also has several limitations. These agents do not affect tumor cell growth selectively over rapidly growing normal cells, leading to high toxicity and side effects. For example, paclitaxel and related taxanes are a very potent class of anticancer drugs first isolated in 1971 (1). Paclitaxel has a unique mechanism of action; it promotes microtubule polymerization, leading to abnormally stable and nonfunctional microtubules. Hence, cells are blocked at the $G_2$-M phase of the cell cycle (2), leading to apoptotic death.

Paclitaxel has clinical efficacy, despite several problems associated with poor solubility and high toxicity. Clinical trials conducted in the late 1980s and early 1990s showed remarkable efficacy against advanced solid tumors such as ovarian and breast cancer (3). More recent clinical studies showed its strong activity against a panel of other tumors (4). Most of the side effects of taxanes occur at rapidly growing tissue such as bone marrow, hematopoietic, and gut epithelia. Because microtubule function is key for neuronal survival, neurotoxicity is also a problem for taxanes (5).

The problem of selectivity can be addressed by using mAbs[3] that target "tumor markers," which are proteins generally overexpressed on the surface of tumor cells. In passive immunotherapy, mAbs can act either as pharmacological agents (6), as adjuvants, or as cytotoxic agents upon fixation of complement (7) and as carriers for large toxins (8) or cytokines (9). However, mAbs are generally poor pharmaceuticals (10) and are poor cytotoxic agents.

Hence, we and others have proposed that chemical coupling of mAbs and chemotherapeutics would allow the delivery of a cytotoxic agent to the tumor cell with reduced side effects. For example, doxorubicin has been coupled to antibodies and peptide carriers (11). However, doxorubicin could still be active in the protein-doxorubicin conjugate, and it would be more desirable to obtain a conjugate that remains inactive until after binding to the target occurs.

To bypass some of the problems above, we aimed to fulfill the following five objectives: (*a*) to chemically conjugate mAbs to the relatively small chemotherapeutic taxanes; (*b*) to afford conjugates that are highly soluble in physiological buffers; (*c*) chemical coupling should not affect mAb targeting function but should result in inactivation of taxane activity; (*d*) after binding to the target receptor mAbs should induce capping and internalization and therefore deliver the conjugate into the tumor cell; and (*e*) the chemical coupling has to allow the release of the taxane in its active form after the antibody-cytotoxic drug conjugate is internalized. This is achieved by coupling the taxane via a low pH-sensitive bond that is cleaved after conjugate internalization and exposure to lysosomal vesicles.

Two target cell surface markers were selected, which correspond to the receptors for nerve growth factor, the p140 TrkA tyrosine kinase high-affinity receptor (12) and the p75 low-affinity receptor (13). TrkA and p75 receptors are expressed on normal cells such as neurons; but at low density, however, they are overexpressed in many cancer cell types. These receptors are useful markers for neuroblastoma, small cell lung carcinoma, B-cell lymphoma, and melanoma (14). mAbs have been developed against those receptors, *i.e.*, anti-p75 mAb MC192 (15) and anti-TrkA mAb 5C3 (16).

We report the synthesis of conjugates paclitaxel-MC192 as an agent to target and kill cells expressing p75 receptors. We also report the synthesis of paclitaxel-rabbit-antimouse antibody as an all-purpose secondary reagent that allows selective tumor targeting with the use of any mouse primary antibody. The paclitaxel-coupled antibodies retain high affinity and specificity after conjugation, and the conjugates delivered the cytotoxic agent in its active form. Paclitaxel-antibody conjugates had *in vitro* cytotoxic activity better than free paclitaxel or free paclitaxel plus free mAb and also showed high selectivity and specificity toward cells expressing the targeted receptors. *In vivo* studies showed that paclitaxel-MC192 conjugate had a good antitumor activity, whereas free drugs had no effect at equivalent concentrations.

These studies will result in an increase or an improvement of the armamentarium and selectivity of cytotoxic agents. Combinations of other chemotherapeutic agents and other ligands using this approach will generate a severalfold increase in the number of antitumor agents.

## MATERIALS AND METHODS

**Synthesis of 2′-Glutaryl-Paclitaxel and Conjugation to Antibodies.** 2′-Glutaryl-paclitaxel was synthesized by mixing 39 μM paclitaxel (Sigma) with 3 μM glutaric anhydride (Sigma), each dissolved in pyridine, for 3 h at room

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[3] The abbreviations used are: mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorter.

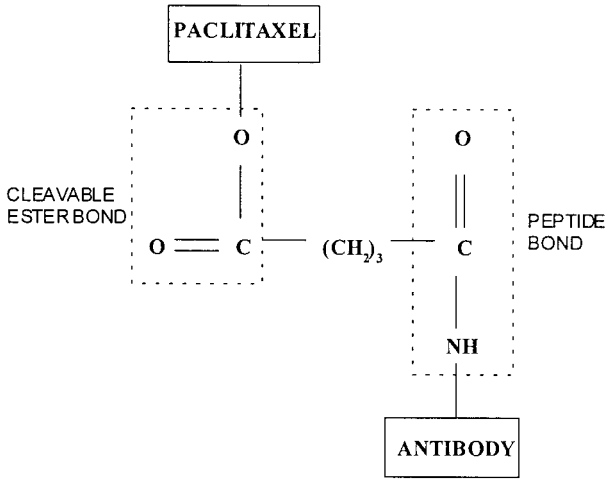


Fig. 1. Structure of paclitaxel-antibody conjugate. Paclitaxel reacted with glutaric anhydride to give 2′-glutaryl-paclitaxel containing a cleavable ester bond. 2′-Glutarylpaclitaxel was then activated by removal of an hydroxyl group with the carbodiimide reaction. The antibody was then bound to activated 2′-glutarylpaclitaxel via its amino group forming a peptide bond.

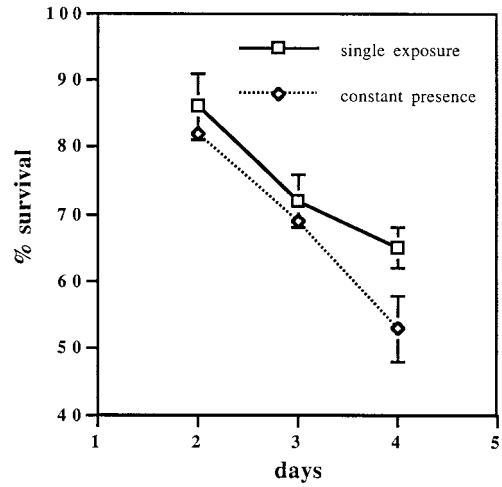


Fig. 2. Paclitaxel cytotoxicity: comparison of dose effectiveness in continuous presence *versus* single exposure to drug. Paclitaxel (40 nM) was added to B104 cells for 30 min. Cells were then cultured for the days indicated without washing (◇, constant presence of paclitaxel) or after washing off the unincorporated paclitaxel (□, single 30-min exposure to paclitaxel). Absorbance (*A*) of MTT assays were quantitated and standardized to untreated cells (100%), using the formula $A_{test}/A_{untreated\ cells} \times 100$ ($n = 4$). Data are representative of three experiments; *bars*, SE. Similar kinetics of survival were obtained with 4-3.6 cells (data not shown).

temperature (17). This reaction forms an ester bond at the C2′ position of paclitaxel (Fig. 1). The solvent was then removed *in vacuo*, and the residue was dissolved in $CHCl_3$ and washed with double-distilled $H_2O$. Purification was achieved by high-performance liquid chromatography on a semipreparative column (Phenomenex); the mobile phase consisted of acetonitrile:water gradient from 35:65 to 75:25 over 50 min.

2′-Glutaryl-paclitaxel (1.334 nmol) was then derivatized with N,N′-carbonyldiimidazole (13.34 nmol; Sigma) for 25 min at 45°C. The carbodiimide reaction activates a carboxylic group on 2′-glutaryl-paclitaxel by removing a hydroxyl. Then, antibody was added slowly over a 20-min period at room temperature at a 2:1 molar ratio of paclitaxel-antibody, and the reaction proceeded for 16 h at 4°C. The reaction forms a paclitaxel-antibody conjugate via formation of a peptide bond with amino groups in the protein (Fig. 1). The solution was then dialyzed for 2 h against water and overnight against PBS. The conjugate can internalize into cells by receptor-mediated and capping mechanisms (18).

**Quantification of Conjugated Paclitaxel.** A known mass of paclitaxel-antibody conjugate was incubated for 48 h at room temperature in 0.1 M acetate buffer (pH 4) to hydrolyze ester bonds. Paclitaxel was then extracted with chloroform and evaporated to dryness. Quantification of this purified paclitaxel was done by analytical high-performance liquid chromatography (Phenomenex) on a mobile phase of acetonitrile:water from 35:65 to 75:25 over 40 min. Known concentrations of paclitaxel were used as standard control. Generally, the measured molar ratio of protein:coupled paclitaxel was 1:1, meaning that 1 molecule of paclitaxel coupled to 1 molecule of antibody. Thus, theoretically, 1 mole of paclitaxel is delivered per mole of internalized antibody.

**Cell Lines.** The B104 cells are a rat neuroblastoma line that expresses p75 receptors ($p75^+$). The 4-3.6 cells are B104 cells stably transfected with human TrkA cDNA ($p75^+$, $TrkA^+$; Ref. 19). NIH 3T3 are mouse fibroblasts that do not express either p75 or TrkA. All cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, L-glutamine, HEPES buffer, and antibiotics.

**Antibodies.** mAb MC192 is a mouse IgG1 antirat p75 mAb (15), and mAb 5C3 is a mouse IgG1 antihuman TrkA mAb (16). MC192 and mAb 5C3 were purified and characterized as described (20) and were used in culture at 1–5 nM, which are near-saturating concentrations for cell surface receptors. The "all purpose" secondary rabbit-antimouse IgG (Sigma) was used in culture at a final concentration of 30 nM.

Table 1 *Efficient target binding by paclitaxel-antibody conjugates*

4-3.6 cells were bound with mAb 5C3 (10 μg/ml), followed by paclitaxel-rabbit-antimouse or intact rabbit-antimouse, and goat-antirabbit-FITC. B104 cells were bound with paclitaxel-MC192 (10 μg/ml) or unconjugated MC192 (10 μg/ml), followed by goat-antimouse-FITC. Background was assessed by replacing the primary mAb with mouse IgG (10 μg/ml). Cells were analyzed (5000/assay) by FACScan and LYSIS II software. The data are mean channel fluorescence of bell-shaped histograms, standardized to maximal staining by unconjugated primary antibody ± SE ($n = 4$).

| | Binding fluorescence | |
|---|---|---|
| Experimental conditions | 4-3.6 cells | B104 cells |
| Background staining | 4 ± 3 | 3 ± 0 |
| Paclitaxel-coupled antibody | 81 ± 28 | 130 ± 7 |
| Intact antibody | 100 | 100 |

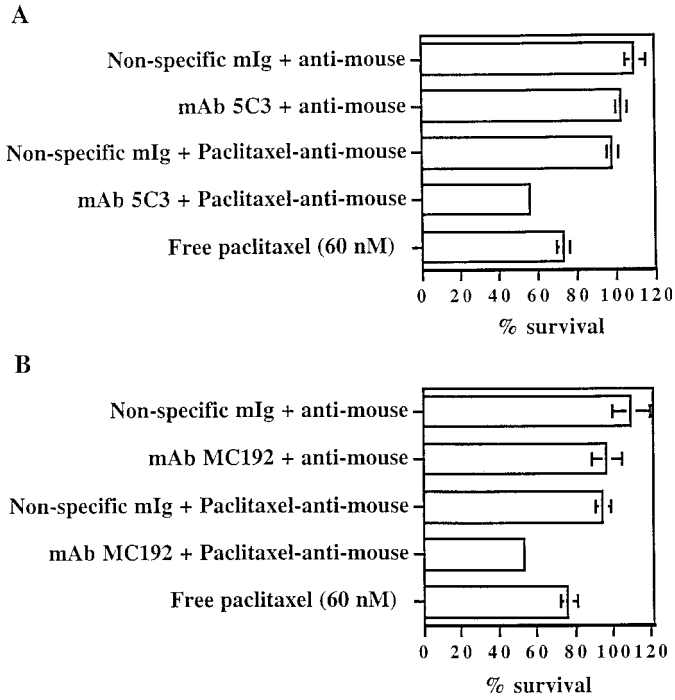


Fig. 3. Cytotoxicity and specificity of paclitaxel-rabbit-antimouse conjugates. 4-3.6 (*A*) and B104 (*B*) cells were cultured with 5 nM specific mAbs (5C3 and MC192, respectively). Each condition was supplemented with 30 nM of either paclitaxel-rabbit-antimouse (test) or rabbit-antimouse antibody (control). The percentage of survival (*bars*, SE) was determined by standardizing untreated cells to 100% ($n = 3$). All points have error bars; some are not seen because they are small. Data are representative of two independent experiments. Similar data were obtained with constant or single-drug exposure.

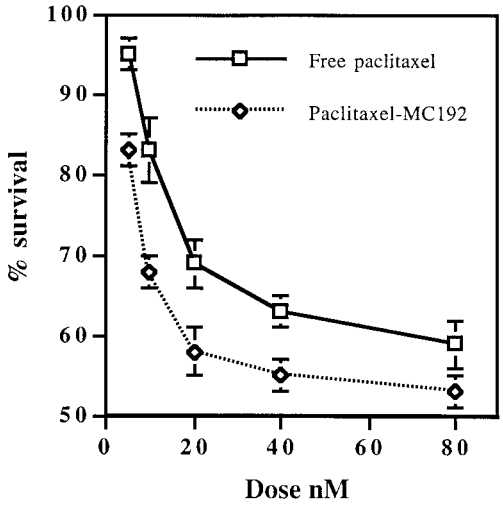


Fig. 4. Comparable dose-dependent cytotoxicity of free paclitaxel and paclitaxel-MC192 conjugate. B104 cells were cultured for 3 days in the constant presence of equivalent molar doses of paclitaxel-MC192 or free paclitaxel. The percentage of survival (*bars*, SE) was determined by standardizing the absorbance (*A*) of untreated cells to 100% ($n = 3$).

**Binding Profiles of the Conjugated Antibodies.** FACScan assays were used to measure the receptor binding properties of the conjugated antibodies. For testing p75 receptor binding by paclitaxel-MC192, B104 cells in binding buffer [BB: HBSS, 0.1% BSAe (BSA), 0.1% $NaN_3$] were incubated with the indicated concentration of either paclitaxel-MC192 (test), intact MC192 (positive control), or mouse IgG (negative control) as primary antibodies, followed by immunostaining with fluoresceinated goat antimouse IgG as described (16). For testing binding of the paclitaxel-rabbit-antimouse conjugate, 4-3.6 cells were incubated as above with saturating mAb 5C3, followed by the paclitaxel-rabbit-antimouse conjugate (test) or rabbit-antimouse antibody (positive control). Cells were then immunostained with fluoresceinated-goat-antirabbit IgG. All data (5000 cells/point) were acquired on a FACScan and analyzed using the LYSIS II program. Data are reported as mean channel fluorescence of bell-shaped histograms.

**Kinetics of Paclitaxel Cytotoxicity: Single Bolus *versus* Constant Exposure.** It is likely that a single bolus of paclitaxel-antibody conjugate would be delivered because affected cells would not synthesize additional target receptor. Therefore, we tested whether a single bolus of paclitaxel would be an effective cytotoxic agent. Cells were exposed to the indicated concentration of paclitaxel for 30 min at 4°C. Then, cells were plated in a 96-well plate (Falcon); this group represents treatment with paclitaxel present in a constant manner. The remaining cells were washed free of excess paclitaxel prior to plating; this group represents a single exposure to paclitaxel. The survival profile of the cells was measured using the tetrazolium salt reagent MTT (Sigma) 48, 72, and 96 h after plating as described (20). Absorbance readings of MTT were done in an EIA Plate Reader model 2550 (Bio-Rad) at 595 nm.

***In Vitro* Cytotoxicity of the Paclitaxel-Antibody Conjugates.** For testing the paclitaxel-MC192 conjugate, cells in 96-well plates (2500–5000 cells/well) were exposed to either MC192, paclitaxel-MC192 conjugates, or controls. Competition of paclitaxel-MC192 cytotoxicity was done by adding a 4-fold molar excess of MC192 antibody. The survival profile of the cells was measured with the MTT assay 72 h after plating.

For testing the "all purpose" rabbit-antimouse reagent, cells were first exposed to primary mouse mAb 5C3, to mAb MC192, or controls (*e.g.*, mouse IgG). Then, paclitaxel-rabbit-antimouse conjugate (test) or rabbit-antimouse antibody (negative control) were added to the cultures. The survival profile of the cells was measured with the MTT assay 48 h after plating.

**Mechanism of Action of Paclitaxel-Antibody Conjugates.** B104 cells were plated, 25,000 cells/well in a 48-well plate (Falcon). Free paclitaxel (80 nM) or paclitaxel-MC192 (40 nM paclitaxel-equivalent) was added to the well, and the cells were incubated for 24 h. Cells were then treated with 0.01% Triton X-100, 0.1% sodium citrate, and 1 $\mu$g DNase-free RNase for 1 h at 0°C. Nuclei were collected after centrifugation. The DNA was labeled with 75 $\mu$l of propidium iodide (1 mg/ml stock) in 400 $\mu$l of FACS buffer. All data (3000 cells/point) were acquired as described above. Paclitaxel release from the conjugate occurs by hydrolysis of the ester bond in the lysosomal compartment (18).

***In Vivo* Tumor Studies.** Nude mice (7 weeks of age, females) were used to test the effect of paclitaxel or conjugates in tumor progression. Single-cell suspensions of B104 cells ($10^5$/mouse) were injected s.c. in the left flank near the rib cage. Tumor growth was monitored daily. After 4 days, the tumor volume in all animals was ~2 $mm^3$. Mice were then randomized, and treatments were initiated in four groups ($n = 5$ in each group). Mice in group 1 received saline; mice in group 2 received free paclitaxel (130 ng); mice in group 3 received free paclitaxel (130 ng) + free MC192 (10 $\mu$g); and mice in group 4 received paclitaxel-MC192 conjugate (65 ng of paclitaxel-equivalent and 10 $\mu$g of MC192-equivalent). All treatments were done by a total of five injections every 2 days (for a total of 10 days). All injections were done i.p. on the right side to prevent direct contact of the agents to the tumor growing s.c. and to assure that systemic circulation of the drugs was achieved. Measurements of tumor volume were taken using calipers every day after treatment for a total of 25 days. The timeline was: day −14, injection of tumors s.c.; days −10, −8, −6, −4, and −2, injections of drugs or controls; and days 0–25, daily measurement of tumor growth.

**Statistical Analysis.** Statistical significance of differences in tumor growth among the different treatment groups was determined by the Student *t* test using SYSTAT 7.0 software. $P < 0.05$ was considered significant.

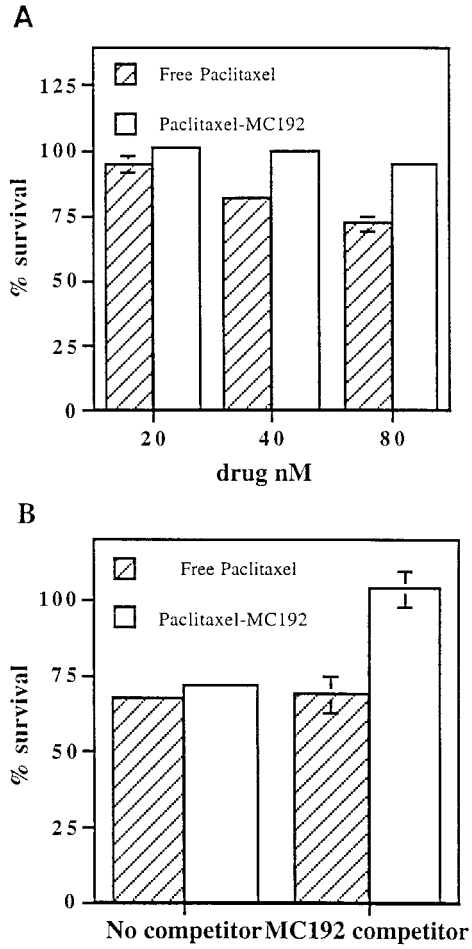


Fig. 5. Paclitaxel-MC192 is target selective and specific toward p75-positive cells. *A*, NIH-3T3 cells were cultured with the indicated molar or molar-equivalent concentrations of drugs. *B*, B104 cells were cultured with free paclitaxel (20 nM) or paclitaxel-MC192 (10 nM paclitaxel-equivalent) in the presence or absence of 40 nM unconjugated MC192 mAb. The percentage of survival (*bars*, SE) was standardized relative to untreated cells (100%; $n = 3$). All points have error bars; some are not seen because they are small. Data are representative of two independent experiments.

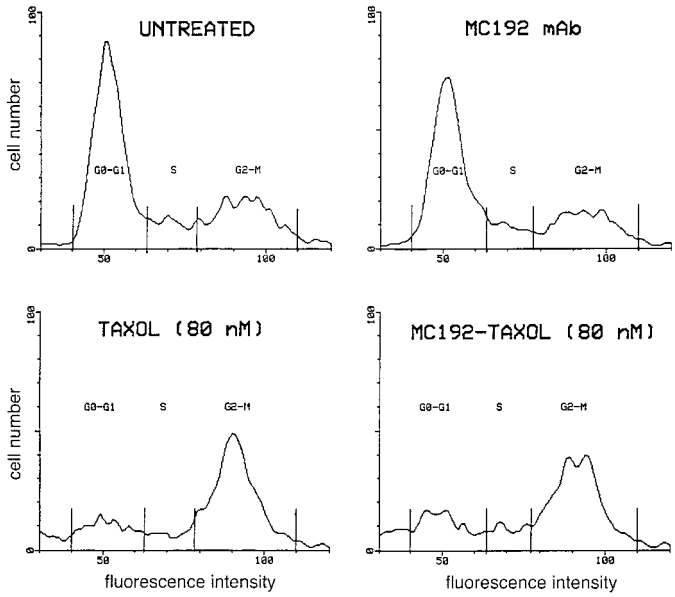


Fig. 6. Paclitaxel-MC192 conjugates and free paclitaxel arrest cells at the $G_2$-M phase of the cell cycle. B104 cells were untreated or treated with 40 nM MC192, paclitaxel-MC192 conjugates (40 nM paclitaxel-equivalent; 40 nM MC192), or 80 nM free paclitaxel. After propidium iodide labeling of DNA, the cell cycle was analyzed by FACScan using LYSIS II software. Cells with <2N DNA (*left* of $G_0$-$G_1$) are apoptotic/dead.

## RESULTS

**Kinetics of Paclitaxel Cytotoxicity: Single Bolus *versus* Constant Presence.** Free paclitaxel is lipophilic and readily penetrates the cell membrane. In contrast, paclitaxel-antibody conjugates penetrate the cell via receptor-mediated internalization. Because it is likely that paclitaxel-antibody conjugate would be delivered as a single bolus (because cells affected would cease synthesis of receptor targets), we tested whether a single short-term exposure to paclitaxel can be effective in killing neuroblastoma B104 cells (Fig. 2). These assays were done at 4°C to allow internalization of drug comparable with that afforded by antibody-mediated delivery of paclitaxel.

The cytotoxic effect of free paclitaxel is generally the same whether the drug is present in the culture throughout or after a single exposure. Comparable killing was verified at several paclitaxel concentrations. However, a single exposure to 20 nM paclitaxel is significantly less effective than constant exposure after 72 and 96 h of culture (data not shown). Likely the amount of drug taken up by the cells after 30 min exposure to 20 nM paclitaxel is sufficient to kill cells over a period of 2 days but not for longer times. These results were encouraging because the cytotoxicity of paclitaxel-antibody conjugates would be similar to that seen after short-term or single exposure to free paclitaxel. Similar data were obtained with 4-3.6 cells (data not shown).

**Binding and Cytotoxicity of Paclitaxel-conjugated Antibodies.** To assess whether paclitaxel conjugation to antibodies affected antibody binding, this property was tested in FACScan assays (Table 1). Conjugated paclitaxel-rabbit-antimouse lost only ~20% of the binding activity compared with unconjugated rabbit-antimouse antibody. The binding activity of conjugated paclitaxel-MC192 was intact, compared with unconjugated MC192. These results indicate that the method used to conjugate paclitaxel to antibody in a 1:1 ratio does not affect significantly the binding properties of the antibodies.

The cytotoxic activity of the "all purpose" paclitaxel-antimouse conjugate was evaluated *in vitro* against neuroblastoma cells (Fig. 3). The paclitaxel-rabbit-antimouse conjugate was active against cells only when a specific mouse primary antibody was present: mAb 5C3 that binds 4-3.6 cells (Fig. 3*A*) and mAb MC192 that binds B104 cells (Fig. 3*B*). Cytotoxicity was better and more selective than equimolar doses of free paclitaxel. In control assays, conjugate paclitaxel-rabbit-antimouse in the presence of a nonspecific primary was not cytotoxic, and the specific primary mAbs in the presence of unconjugated rabbit-antimouse were not cytotoxic. Similar analysis using paclitaxel-MC192 conjugates also showed better activity and selectivity than free paclitaxel at equimolar concentrations (Fig. 4).

These data suggest that the paclitaxel-rabbit-antimouse conjugate was active by binding to the specific primary antibody, whereas paclitaxel-MC192 conjugates were active by directly targeting p75 receptors. Presumably, the conjugates internalized and released the cytotoxic agent inside the cells. Because only a fraction of paclitaxel-antibody conjugates can internalize via the targeted receptor, the data suggest that conjugates may be significantly much better at cell killing than free paclitaxel, possibly because of improved transport or penetration. Paclitaxel release after hydrolysis of the conjugate could not be measured directly *in vivo* because of technical limitations.

**Selectivity and Specificity of Paclitaxel-MC192 Conjugates.** The selectivity of paclitaxel-MC192 was evaluated using cells that do not express p75 (Fig. 5*A*). The results show that the conjugate was inactive, whereas free paclitaxel exhibited dose-dependent cytotoxicity. These results suggest that the activity of paclitaxel-MC192 conjugates is selective toward cells expressing p75 receptors. The specificity of paclitaxel-MC192 conjugate was investigated by ligand competition (Fig. 5*B*). At 10 nM paclitaxel-MC192 conjugate (10 nM paclitaxel-equivalent), there is efficient killing of B104 cells. Concomitant addition of 40 nM MC192 blocks cytotoxicity by competing for the p75 receptor target. In contrast, addition of 40 nM nonspecific mouse IgG does not affect the activity of paclitaxel-MC192 conjugates. Cold competition of paclitaxel-MC192 indicates that death is mediated specifically via p75 receptors. Furthermore, free paclitaxel had the same cytotoxicity whether or not 40 nM of mouse IgG or 40 nM of MC192 antibody were added to the cultures.

Because some antibodies increase free drug-mediated killing compared with drugs alone, we investigated whether MC192 had a pharmacological role as adjuvant. MC192 mAb or mouse IgG did not enhance or decrease the cytotoxicity of various concentrations of free paclitaxel (data not shown). Similar data were obtained with 60 nM free paclitaxel cultured with increasing doses of antibody (data not shown). These results indicate that MC192 did not have a pharmacological role and suggest that MC192 acts only as a carrier and does not contribute to the cytotoxicity of the conjugate *in vitro*.

**Cytotoxic Mechanism of Paclitaxel-MC192 Conjugates.** To assess whether the mechanism of action of paclitaxel-MC192 conjugates is the same as free paclitaxel, cell cycle analysis was done in FACScan assays (Fig. 6). The data show that paclitaxel-MC192 conjugates arrest cells in the $G_2$-M phase of the cell cycle, which is consistent with the mechanism of action of free paclitaxel. A $G_2$-M arrest leads to apoptosis in these cells. MC192-treated cells cycle similar to untreated control, indicating no effect by the antibody.

***In Vivo* Activity of Paclitaxel-MC192.** The antitumor activity of paclitaxel-MC192 was evaluated *in vivo* against neuroblastoma xenografted in nude mice (Fig. 7). The results show that the conjugate was effective in reducing tumor growth compared with the control (HBSS; *t* test, $P < 0.05$; Fig. 7*C*), whereas paclitaxel alone or in combination with MC192 were not able to do so (Fig. 7, *A* and *B*). Moreover, the conjugate prolonged the survival of the mice on average by ~30% compared with free paclitaxel (data not shown).

## DISCUSSION

We have shown that mAb MC192 and mAb 5C3, ligands for p75 and trkA receptors, respectively, can be used as carriers for paclitaxel

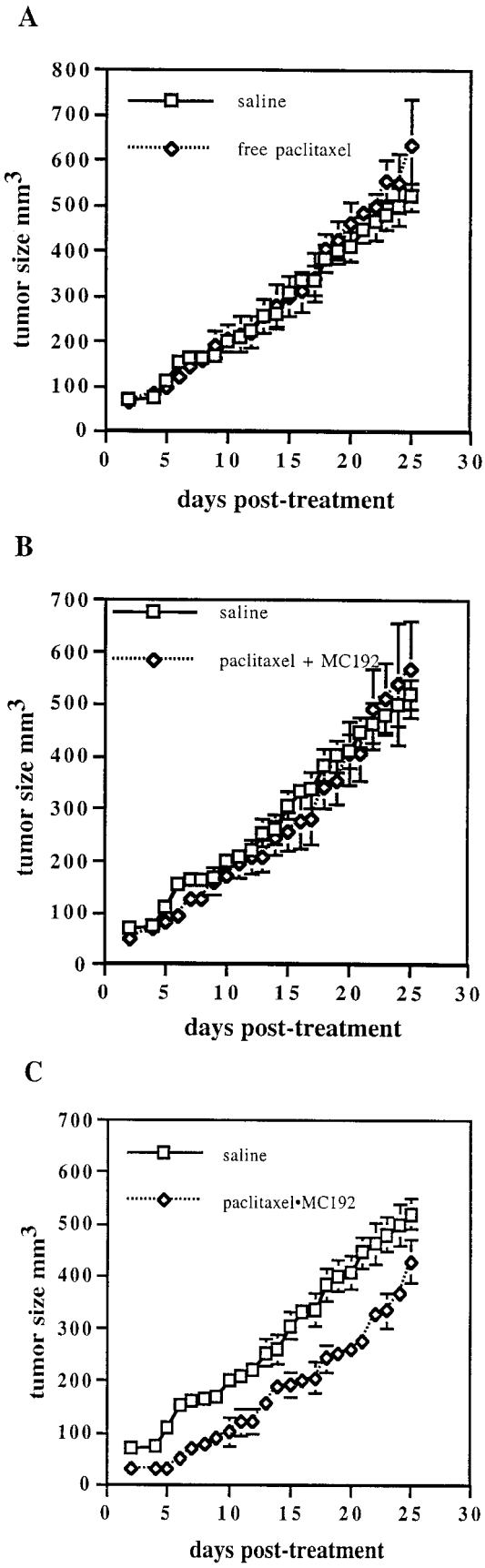


Fig. 7. Paclitaxel-MC192 is efficient at reducing tumor growth and at increasing survival *in vivo*. Tumor sizes of mice that received five treatments every 2 days of saline (*A–C*); 130 ng of free paclitaxel (*A*), 130 ng of free paclitaxel + free MC192 (10 $\mu$g; *B*); or paclitaxel-MC192 (65 ng paclitaxel-equivalent and 10 $\mu$g MC192-equivalent; *C*) are shown. All points have error bars; some are not seen because they are small.

to afford efficient and specific tumor toxicity. We also show that an "all purpose" targeting agent can be developed by paclitaxel conjugation of anti-immunoglobulin secondary antibodies.

**Kinetics of Paclitaxel Cytotoxicity.** Because the cytotoxicity of paclitaxel-antibody conjugates was expected to be similar to that seen after short-term or single exposure to free paclitaxel, we first assessed whether a single dose of paclitaxel could be efficient at killing the cells. We demonstrated that paclitaxel cytotoxicity is the same after 48 h, whether a constant or a single dose is given *in vitro*. This finding underlines the clinical experience of paclitaxel delivery, which is often delivered as a single bolus every few weeks (4), unlike many other chemotherapeutics that are most effective when delivered at low doses over prolonged periods.

**Criteria for Conjugation.** We set out to fulfill several criteria that would improve the therapeutic index of paclitaxel: (*a*) to chemically conjugate mAbs to taxanes; (*b*) to afford conjugates that are highly soluble in physiological buffers; (*c*) chemical coupling should not affect mAb targeting function, but should result in a prodrug; and (*d*) after binding to the target receptor mAbs should induce internalization and release the drug. All of these criteria were fulfilled.

Paclitaxel was linked via its most reactive hydroxyl group (C2′ position) to antibodies as carriers. This esterification leads to paclitaxel inactivation because this position is crucial for tubulin binding (21); thus, the conjugate is a prodrug. The binding activity of the antibodies were essentially preserved after coupling; only ~20% loss of binding was observed for the "all purpose reagent" and no loss for MC192. After hydrolysis of the conjugate, active paclitaxel is released in sufficient amounts to kill cells. As expected, the conjugates arrest the cells in prophase, such as paclitaxel does.

**Improved Efficacy.** The cytotoxic activity of the conjugates was better than that of free paclitaxel. This may be attributable to better transportation, penetration, and accumulation of the drug inside the cells. Moreover, conjugate cytotoxicity related well with the density of target receptors on the cell lines. Although it has been suggested that in certain systems ligand-bound p75 receptors may activate ceramide pathways and proapoptotic signals (22), the carrier itself had no direct pharmacological role. Hence, cytotoxicity is only attributable to paclitaxel. It would be very interesting to use carriers that are cytotoxic such as anti-HER-2 mAbs (23); they may be better agents because of a synergistic or additive effect.

**Improved Selectivity.** No binding of the conjugates was observed in cells that do not express the target receptors. We selected the paclitaxel-MC192 conjugate for *in vivo* experiments because it was more suitable than the "all purpose" paclitaxel-rabbit-antimouse conjugate. The *in vivo* experiments confirmed our *in vitro* findings. The paclitaxel-MC192 conjugate had a significant antitumor activity against cells expressing p75 receptor in the experimental model used, and we observed a delay in tumor growth compared with other groups.

The efficacy of the conjugate compared with free drug was much more evident *in vivo* than *in vitro*, probably because *in vivo* the conjugate was concentrated at the tumor site. The effective concentration of conjugate tested *in vivo* was ~3.5 nM, whereas free paclitaxel was not effective at this dose. Because the effective concentration of taxanes in humans is in the millimolar range, the therapeutic index of the conjugate is improved severalfold. Futhermore, we demonstrated appropriate *in vivo* systemic distribution and pharmacokinetics for the conjugate.

It was not possible to assess the toxicity of the conjugate compared with that of free paclitaxel because of limitations in supply. Nevertheless, we did not observe any obvious toxicity in the treated animals, and we speculate that the paclitaxel-MC192 conjugate would be much less toxic than free paclitaxel because it spares non-target-expressing cells.

All of the reasons mentioned above make the paclitaxel-MC192 conjugate a potential candidate for the treatment of p75-expressing

tumors. The conjugates are not only better cytotoxic agents than free paclitaxel, but they are also highly water-soluble, which is a great advantage considering the severe hypersensitivity reactions experienced by paclitaxel-treated patients. The properties of the conjugates may make them interesting therapeutic agents to add to the chemotherapeutic armamentarium. Indeed, the Food and Drug Administration has recently approved an antibody-directed cytotoxic antibiotic for the treatment of acute myeloid leukemia.

Paclitaxel is frequently given in combination with antibodies but not physically bound to them; here, we report the first synthesis of paclitaxel-antibody conjugates. A general method is proposed to selectively target cancer cells by concentrating cytotoxic drugs at the tumor site and inside the tumors. Furthermore, it would be interesting to couple cytotoxic drugs to small peptidic (10) or nonpeptidic ligands (24) of tumor markers to overcome obstacles (such as proteolysis, immunogenicity, and poor penetration of solid tumors) inherent to antibodies and proteins (14) when used as therapeutics.

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