Enhanced Antitumor Activity of Paclitaxel in Combination with the Anticarcinoma Immunoconjugate BR96-Doxorubicin

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ABSTRACT
The efficacy of chemotherapy has been improved by regimens that combine several cytotoxic drugs with different mechanisms of action and/or different dose-limiting toxicities. Here we demonstrate clearly, and for the first time, that combined therapy using an anticarcinoma immunoconjugate, BR96-doxorubicin, and the cytotoxic drug paclitaxel results in a significant increase in antitumor activity over that of either agent alone. Synergistic activity was seen at doses of BR96-doxorubicin that were minimally active as single agents. A dramatic increase in regression rates was seen when a regimen that combined BR96-doxorubicin and paclitaxel was used to treat both paclitaxel-sensitive and paclitaxel-insensitive carcinomas. Importantly, combined therapy resulted in increased antitumor activity against lung, colon, and breast tumors xenografted in athymic mice and large, paclitaxel-insensitive colon tumors xenografted in athymic rats that also express the Lewisy target antigen in normal tissues.

INTRODUCTION
MAbs3 and MAb-directed immunoconjugates and immuno-toxins have shown limited clinical efficacy when evaluated as single agents in patients with advanced solid tumors (1, 2). A variety of factors, including tumor heterogeneity and the emergence of drug-resistant populations, limit the efficacy of cancer therapy. In addition, the physical barriers of solid tumors, including elevated interstitial pressure and a heterogeneous and reduced functional vasculature (3), contribute to the poor tumor penetration and minimal efficacy seen when MAb-directed therapies are used in patients with advanced disease. In contrast to the results seen in patients with advanced solid tumors, MAb-directed therapy has shown utility for patients with lymphomas (4–6) and patients with minimal residual disease following resection of Dukes’ C colon carcinoma (7).

We previously described preclinical studies with BR96-doxorubicin, an anticarcinoma immunoconjugate (8, 9) that binds to a Ley-related tumor-associated antigen abundantly expressed (>200,000 molecules/cell) on the majority of human carcinomas. In preclinical models, treatment with BR96-doxorubicin at doses of ≥100 mg/kg resulted in cures of established tumors in athymic mice or rats xenotransplanted with human lung, breast, or colon carcinomas (1, 8) and of syngeneic colon tumors growing either s.c. or in the liver of immunocompetent rats (10). The cures in rats occurred although the Ley target antigen was expressed by normal cells in the GI tract (8, 10).

BR96-doxorubicin was evaluated in phase I trials in patients with advanced disease at doses of up to 875 mg/m2 (26 mg/m2 doxorubicin) every 3 weeks. Localization of both the BR96 MAb and doxorubicin were seen in patient tumor biopsies 24 h after administration of BR96-doxorubicin. However, of the 66 patients treated with at least two courses of BR96-doxorubicin, the combined rate of tumor regressions and disease stabilization was 35% (11), with only 2 patients achieving a partial tumor regression. There was no indication of cardiotoxicity; however, acute dose-related GI toxicity limited further escalation of the dose of BR96-doxorubicin. Although the BR96-doxorubicin conjugate demonstrated clinical evidence of biological activity, the doses of conjugate that could be administered safely were not sufficient to achieve and maintain the intratumoral concentrations of doxorubicin necessary to achieve regression of advanced tumors.

Substantial improvements in therapeutic efficacy have been seen for treatment regimens that combine multiple cytotoxic drugs with different mechanisms of action. For example, recent clinical studies have shown that regimens that use doxorubicin in combination with paclitaxel (Taxol®; Bristol-Myers Squibb Company, Princeton, NJ), produce increased regression rates relative to either drug used alone (12–14). In addition, MAbs to the growth factor receptors epidermal growth factor receptor and HER2/neu have been shown to enhance the antitumor activity of cytotoxic drugs such as cisplatin (15), doxorubicin (16, 17), and paclitaxel (17) in preclinical models. Recent studies have extended these observations to the clinic; MAbs to HER2/neu used in combination with cisplatin (18) or paclitaxel (19) have shown improved response rates relative to single agent therapy.

In the studies described here, we evaluated whether administration of BR96-doxorubicin at doses that were minimally effective as a single agent could be used to enhance the activity of drugs such as paclitaxel. These studies demonstrate clearly...
and for the first time that combined therapy with BR96-doxorubicin and paclitaxel improves efficacy relative to either agent alone at doses of conjugate in the range expected to be tolerated clinically. Importantly, synergistic activity was seen for BR96-doxorubicin in combination with paclitaxel when evaluated against both paclitaxel-sensitive and -insensitive tumors and at doses of BR96-doxorubicin that were minimally active as single agents.

MATERIALS AND METHODS

MAbs and Conjugates. BR96-doxorubicin conjugates containing a thiol linker to the MAb and an acid-labile hydrazide link to doxorubicin were produced as described (8, 9) with a drug to MAb molar ratio of 8:1. Conjugates contained <5% free doxorubicin or doxorubicin linker and retained >95% of the original MAb binding activity.

Human Carcinoma Lines. Four human tumor lines of three histological types that express the BR96-defined antigen were evaluated: L2987, a lung adenocarcinoma isolated from a pleural effusion; MCF7, an estrogen-dependent breast carcinoma from the American Type Culture Collection; RCA, a colon carcinoma obtained from M. Brattain (Medical College of Ohio, Toledo, Ohio); and LS174T, a colon carcinoma from the American Type Culture Collection.

Human Tumor Xenograft Models. Human tumor xenografts were established s.c. in female athymic (nude) mice or athymic (Rowett strain) nude rats (Harlan Sprague Dawley, Indianapolis, IN) as described (8). MCF7 breast tumors require supplemental estrogen, which was provided as a s.c. β-estradiol pellet (Innovative Research of America, Toledo, OH). Tumors were measured twice weekly, and size was calculated as 0.5 (length × width²). There were 8–10 mice or 6–8 rats per control or treatment group. Data are presented as mean tumor size ± SE. Tumor responses are defined as: PR, a decrease in size to ≤50% of initial size; CR, a tumor that regressed completely and was not palpable for a period of time equal to the tumor doubling time; and durable CR, a tumor that regressed completely and remained regressed for a period of time equivalent to ≥10 times the tumor doubling time.

Therapy. Treatments were administered i.v. every 2 days for a total of five injections. In combination studies, paclitaxel and BR96-doxorubicin or unconjugated MAb BR96 were administered on alternating days, with BR96-doxorubicin or MAb BR96 therapy started before paclitaxel. Doses are given as mg/kg/injection with BR96-doxorubicin doses reported as mg/kg/injection of MAb. The MTD of paclitaxel in mice was 25–30 mg/kg/injection in mice that were not supplemented with estrogen; however, it was lower (20 mg/kg/injection) in estrogen-supplemented mice. The MTD of paclitaxel in athymic rats was 2 mg/kg/injection.

Flow Cytometry Analysis. Exponentially growing monolayers of L2987 lung or RCA colon carcinoma cells were incubated for 24 h in 10% FCS/RPMI 1640 with BR96-doxorubicin, unconjugated BR96 MAb, doxorubicin, human IgG, or PBS (untreated control). Cells were harvested (trypsin-EDTA), washed, and fixed in 80% ethanol (20°C for 2 h). After centrifugation, cells were resuspended in staining solution containing 50 μg/ml propidium iodide and 0.1% DNase-free RNase A (PharMingen, San Diego, CA) and incubated at room temperature (1 h). The DNA content of triplicate samples was determined (FACScan; Becton Dickinson, Palo Alto, CA), and 10,000 cells were analyzed per sample. The percentage of cells in each phase was calculated with Cell Quest 1.2 (Becton Dickinson) software.

RESULTS

Effects of Combined Therapy with BR96-Doxorubicin and Paclitaxel on Paclitaxel-sensitive Human Breast and Lung Tumor Xenografts. The combined activity of BR96-doxorubicin and paclitaxel on MCF7 breast tumor xenografts is shown in Fig. 1. Paclitaxel, administered at its MTD (20 mg/kg in estrogen-supplemented mice), resulted in significant tumor growth inhibition and induced 33% CRs; however, tumors regrew after therapy was completed (Fig. 1A). BR96-doxorubicin, administered at a dose of 70 mg/kg, resulted in significant tumor growth inhibition and induced 22% CRs and 22% PRs. The antitumor activity of BR96-doxorubicin was dose dependent; BR96-doxorubicin administered at a dose of 17 mg/kg did not produce either a tumor growth delay or regressions. To evaluate the effect of combined therapy, 17 mg/kg of BR96-doxorubicin, a dose that was ineffective as a single agent, was used in combination with the MTD of paclitaxel. Combination therapy with BR96-doxorubicin and paclitaxel resulted in a significant increase in tumor growth inhibition relative to BR96-doxorubicin (P < 0.01, day 22) or paclitaxel (P < 0.03, day 41) administered alone. Combined therapy also increased the regression rate (66% CRs and 11% PRs for the combination relative to 0% CRs for BR96-doxorubicin and 33% CRs for paclitaxel). The antitumor activity seen when BR96-doxorubicin (17 mg/kg) was used in combination with paclitaxel was better than that achieved with a 4-fold higher dose (70 mg/kg) of BR96-doxorubicin used alone. We next evaluated whether BR96-doxorubicin, administered at a suboptimal dose, enhanced the efficacy of an inactive dose of paclitaxel (Fig. 1B, C–D). Included in these studies were treatment arms in which unconjugated MAb BR96 or doxorubicin were combined with paclitaxel at doses equivalent to that of BR96. Paclitaxel at a dose 12 mg/kg (equivalent to 60% of its MTD in estrogen-supplemented mice) did not produce either a significant delay in tumor growth or tumor regressions (Fig. 1B). BR96-doxorubicin, at a dose of 70 mg/kg produced a significant (P < 0.05, day 36) tumor growth delay and 40% PRs. When these doses of BR96-doxorubicin and paclitaxel were combined, the antitumor activity of the combination (50% CRs and 30% PRs) was superior (P < 0.05, day 44) to that of either BR96-doxorubicin or paclitaxel used as single agents. As shown in Fig. 1C, treatment with an equivalent dose of MAb BR96 (70 mg/kg) did not inhibit tumor growth. However, MAb BR96 in combination with paclitaxel was more active than paclitaxel alone, inducing 30% CRs and 50% PRs. In the MCF7 model, combined therapy with BR96-doxorubicin and paclitaxel produced a modest increase in tumor growth delay and tumor regression rates relative to combined MAb BR96 plus paclitaxel; however, this difference was not statistically significant. Treatment with 2 mg/kg doxorubicin, the conjugate-equivalent dose, produced a tumor growth delay but no regressions (Fig. 1D), whereas combining this dose of doxorub-
bicin with paclitaxel (12 mg/kg) resulted in better efficacy (20% CRs and 50% PRs) than seen for treatment with either doxorubicin or paclitaxel alone.

The activity of BR96-doxorubicin and paclitaxel against L2987 lung tumor xenografts is presented in Fig. 2. The MTD of paclitaxel (30 mg/kg) resulted in significant tumor growth inhibition and induced 37.5% PRs; however, tumors regrew after therapy was completed (Fig. 2A). BR96-doxorubicin administered at 70 mg/kg or paclitaxel at 12 mg/kg resulted in complete tumor regression (Fig. 2B). BR96-doxorubicin administered at 70 mg/kg (○) or paclitaxel at 12 mg/kg (□) was administered on days 13, 15, 17, 19, and 21. Combined therapy (□□) included BR96-doxorubicin (17 mg/kg on days 13, 15, 17, 19, and 21) and paclitaxel (20 mg/kg on days 14, 16, 18, 20, and 22). Control animals (●) were not treated. B–D, treatment began 14 days after implant, when tumors were 100 mm³ in size. There were nine mice per group. B, BR96-doxorubicin administered at 70 mg/kg (○) or paclitaxel at 12 mg/kg (□) was administered on days 14, 16, 18, 20, and 22. Combined therapy (□□) included BR96-doxorubicin (70 mg/kg on days 14, 16, 18, 20, and 22) and paclitaxel (12 mg/kg on days 15, 17, 19, 21, and 23). Control animals (●) were not treated. C, BR96 at 70 mg/kg (○) or paclitaxel at 12 mg/kg (□) was administered on days 14, 16, 18, 20, and 22. Combined therapy (□□) included BR96 (70 mg/kg on days 14, 16, 18, 20, and 22) and paclitaxel (12 mg/kg on days 15, 17, 19, 21, and 23). Control animals (●) were not treated. D, doxorubicin at 2 mg/kg (○) or paclitaxel at 12 mg/kg (□) was administered on days 14, 16, 18, 20, and 22. Combined therapy (□□□) included doxorubicin (2 mg/kg on days 14, 16, 18, 20, and 22) and paclitaxel (12 mg/kg on days 15, 17, 19, and 23).

Effects of Combined Therapy with BR96-Doxorubicin and Paclitaxel on Paclitaxel-resistant Human Colon Tumor Xenografts. Studies were also done to determine whether combining BR96-doxorubicin therapy with that of paclitaxel...
BR96-doxorubicin increases the population of cells in G2-M. Studies with synchronized populations have demonstrated that cells are most sensitive to paclitaxel-induced apoptosis during the G2-M and G0-G1 transitions of the cell cycle (20). In the studies described here, we evaluated whether changes in cell cycle distribution occurred as a consequence of exposure to BR96-doxorubicin. The cell cycle distribution following a 24-h exposure of L2987 lung carcinoma cells to BR96-doxorubicin (0.25 μM BR96, 2 μM doxorubicin), 2 μM doxorubicin, 0.25 μM BR96, or 0.25 μM human IgG is shown in Fig. 4. Exposure to BR96-doxorubicin significantly (P < 0.002) increased the percentage of cells accumulating in G2-M (71.7 ± 0.19%) relative to untreated cells (15.24 ± 0.3%). The G0-G1 population was significantly (P < 0.001) reduced following treatment with BR96-doxorubicin, whereas the percentage of cells in S phase was not changed relative to control or IgG. Exposure to an equivalent concentration of doxorubicin resulted in a significant (P < 0.002) increase in the percentage of cells in G2-M, (29.73 ± 0.65%) relative to untreated or IgG-treated cells. This was accompanied by a significant (P < 0.002) decrease of cells in G2-M and a significant (P < 0.002) increase in the percentage of cells accumulating in S phase. In contrast, treatment with BR96 did not change cell cycle distribution relative to untreated control or an equivalent concentration of IgG. A similar change in cell cycle distribution resulting in an increased proportion of cells in the G2-M phase was also observed on RCA colon carcinoma cells treated with BR96-doxorubicin (data not shown).
In the studies described here, we demonstrate clearly and for the first time that combined therapy with BR96-doxorubicin and paclitaxel resulted in a dramatic increase in antitumor activity against human tumor xenografts in athymic mice and rats. BR96-doxorubicin at doses that were not active as single agents enhanced the activity of paclitaxel, producing a significant increase in the tumor regression rate over that seen with an equivalent dose of paclitaxel alone. The effect of combined therapy with BR96-doxorubicin and paclitaxel was seen in four tumor xenografts of three histological types: breast, lung, and colon carcinoma. In each case, a significant increase in antitumor activity was seen for the combination relative to equivalent doses of BR96-doxorubicin and paclitaxel administered alone. The antitumor activity achieved with combined paclitaxel and BR96-doxorubicin therapy was seen both when paclitaxel was administered at its MTD and when it was administered at suboptimal doses. The use of BR96-doxorubicin in combination with paclitaxel resulted in increased antitumor activity against paclitaxel-sensitive lung (L2987) and breast (MCF7) tumor xenografts. Importantly, combined treatment with BR96-doxorubicin and paclitaxel also resulted in increased antitumor activity against paclitaxel-insensitive colon tumors (RCA and LS174T). Antitumor experiments in athymic rats serve as a model for clinical therapy because rats express the Ley antigen on normal cells of the GI tract (8). The studies described here (Fig. 3) demonstrate that combined therapy with paclitaxel and BR96-doxorubicin, administered at a dose equivalent to 20% of its MTD, produced durable CRs of large (500 mm$^3$) tumors in the absence of toxicity even when the target antigen was expressed on cells of normal tissues.

Clinical regimens that combined paclitaxel with doxorubicin have been shown to be more effective in treating advanced breast cancer than regimens that used either drug alone (12–14). In addition, MAbs to the growth factor receptors epidermal growth factor receptor and HER2/neu have been shown to
enhance the antitumor activity of cytotoxic drugs such as cisplatin (15), doxorubicin (16, 17), and paclitaxel (17) in preclinical models. Recently, these observations have been extended to the clinic: MAbs to HER2/neu used in combination with cisplatin (18) or paclitaxel (19) have shown improved response rates relative to the drugs or MAbs used as single agent therapies.

In the studies presented here, we show that treatment with the immunoconjugate BR96-doxorubicin in combination with paclitaxel was more effective than either agent alone against both paclitaxel-sensitive and paclitaxel-insensitive tumors. Potentiation of the antitumor activity of paclitaxel was best achieved with the BR96-doxorubicin conjugate; unconjugated MAb BR96 in combination with paclitaxel did not increase antitumor activity to the extent seen with BR96-doxorubicin. The mechanism(s) responsible for the potentiation of antitumor activity seen when BR96-doxorubicin was combined with paclitaxel are not clear, and specific signal transduction events mediated by BR96-doxorubicin may need to be identified to further understand these mechanism(s). In this regard, no evidence of Bcl-2 or Raf-1 phosphorylation, two events proposed to be associated with paclitaxel-mediated apoptosis (21) was observed after MCF7 cells were treated with different concentrations of BR96-doxorubicin (0.1–3.2 μM) (data not shown). In addition, BR96-doxorubicin was shown to potentiate the activity of paclitaxel against tumors bearing either wild-type p53 (MCF7 and LS174T) or mutant p53 genes (L2987 and RCA).4

Paclitaxel binds to tubulin microtubules and stabilizes them by inhibiting depolymerization (22, 23). Studies with synchronized populations demonstrated that cells are most sensitive to paclitaxel-induced apoptosis during the G2-M phase of the cell cycle (20, 23). In the studies presented here, we show that exposure of human lung carcinoma cells to BR96-doxorubicin resulted in a significant increase in the percentage of cells in G2-M. Exposure to equivalent doxorubicin also resulted in an increase in the percentage of cells accumulating in G2-M, but to a lesser extent than seen with BR96-doxorubicin. In contrast, exposure to BR96 MAb did not result in a change in cell cycle distribution. The increased proportion of cells in G2-M phase following treatment with BR96-doxorubicin may be associated with enhanced sensitivity of cells to paclitaxel-induced apoptosis. A similar change in cell cycle distribution was seen for paclitaxel-insensitive RCA carcinoma cells (data not shown).

It is unlikely that the enhanced antitumor activity seen with combined BR96-doxorubicin and paclitaxel therapy is due to an additive effect of drugs with different mechanisms of action because potentiation was also seen when the combination was evaluated against tumors that were not sensitive to paclitaxel. Treatment with free doxorubicin, rather than BR96-doxorubicin, alone or in combination with paclitaxel did not produce comparable antitumor effects. Interestingly, preliminary data demonstrate a similar increase in antitumor activity when BR96-doxorubicin was used in combination with cisplatin, mitomycin C, or doxorubicin.5

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4 A. B. Bianchi, unpublished results.

5 P. A. Trail, unpublished results.
suggesting that BR96-doxorubicin may have utility as a chemopotententiating agent in multiple therapeutic regimens.

In summary, our data demonstrate that BR96-doxorubicin significantly enhanced the antitumor activity of paclitaxel in several preclinical models of human carcinoma. Increased antitumor activity was seen when BR96-doxorubicin was used in combination with paclitaxel against paclitaxel-sensitive breast and lung tumor xenografts. Importantly, combined therapy with BR96-doxorubicin and paclitaxel also resulted in a significant increase in activity when evaluated against paclitaxel-insensitive colon tumor xenografts in athymic mice and rats.

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REFERENCES


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