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Marked enhancement *in vivo* of paclitaxel's (taxol's) tumor-regressing activity by ATP-depleting modulation

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Paclitaxel alone is active against the CD8F1 murine spontaneous mammary cancer, and when administered following an ATP-depleting combination of *N*-(phosphonacetyl)-L-aspartate (PALA) + 6-methylmercaptopurine riboside (MMPR) + 6-aminonicotinamide (6-AN) (PMA) produced significantly enhanced partial tumor regressions over that produced by either paclitaxel alone at the maximal tolerated dose (MTD), or by the PMA drug combination alone, against advanced, first passage spontaneous murine breast tumors. The anticancer activity of paclitaxel is due to enhancement and stabilization of microtubule polymerization. Pertinently, microtubule disassembly (an ATP-dependent process) is known to sharply decrease in the presence of ATP depletion. Thus, the dramatic therapeutic enhancement observed with paclitaxel in combination with PMA is in agreement with biochemical expectations, since PMA has been shown to deplete ATP in CD8F1 tumor cells. The augmented therapeutic results were obtained with approximately one-third the MTD of paclitaxel as a single agent and suggest the potential clinical benefit of more effective treatment with lesser amounts of drug.

Key words: ATP depletion, biochemical modulation, murine mammary tumors, paclitaxel, tumor regression.

Introduction

The antimicrotubule agent, paclitaxel, has demonstrated significant antineoplastic activity in patients with refractory ovarian cancer, refractory breast cancer, non-small cell lung cancer, head and neck cancer, and other types of cancers.[1] Its broad antitumor activity and novel mechanism of action have generated great interest. Paclitaxel enhances and stabilizes microtubule polymerization, induces extensive microtubule bundles and multinucleation of cells, blocks mitosis, and kills cells by apoptosis.[2–7] Microtubule disassembly, however, is an ATP-dependent process that sharply decreases in the presence of energy metabolism inhibitors that reduce the intracellular ATP pools.[8–10] For example, cyclocreatine, which reduces ATP availability, has been reported[11] to produce synergistic killing of tumor cells *in vitro* in combination with paclitaxel. ATP depletion apparently inhibits microtubule phosphorylation, thereby increasing microtubule stability and suppressing microtubule dynamics.[8–10] Against this background, it seemed logical to evaluate the administration of PMA prior to paclitaxel.

PMA, a drug combination designed to primarily deplete cellular energy (i.e. ATP) in tumor cells, has been shown to have preclinical anticancer activity *in vivo*,[12,13] and when administered prior to a variety of anticancer agents, or prior to radiotherapy, produces significantly greater tumor-regressing activity *in vivo* than that achieved by either the PMA combination alone or the maximal tolerated dose (MTD) of the anticancer effector agent(s) alone on CD8F1 murine advanced breast tumors.[12–15] The biochemical rationale and the supporting biochemical data showing that PMA significantly lowers ATP levels (e.g. to 30% of saline-treated control tumors) in CD8F1 murine breast tumors have been presented.[12,13,16]

This publication reports that paclitaxel alone is active against the CD8F1 tumor, that the administration of the *N*-(phosphonacetyl)-L-aspartate (PALA) + 6-methylmercaptopurine riboside (MMPR) + 6-aminonicotinamide (6-AN) (PMA) drug combination prior to paclitaxel significantly enhanced antitumor activity over that produced by either paclitaxel alone at MTD, or the PMA drug combination alone, and that only one-third the MTD dose of paclitaxel (in

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combination with PMA) is required for this greater level of antitumor activity.

Materials and methods

CD8F1 murine breast tumor system

CD8F1 hybrid mice bearing single spontaneous, autochthonous breast tumors were selected from our colony which has been described previously[17,18] and which was included in the murine tumor testing panel of the National Cancer Drug Screening Program.[19,20] For each first passage tumor transplant experiment, a tumor cell brei, prepared by pooling three or four spontaneously arising CD8F1 breast tumors, was transplanted subcutaneously into syngeneic 3-month-old BALB/c × DBA/8 F_1 (hereafter called CD8F1) mice. In approximately 3–4 weeks, when transplanted tumors were well advanced and measurable, all tumor transplants were measured and the tumor-bearing mice were distributed among experimental groups (10 tumor-bearing mice/group) so that mice carrying tumors of approximately equal weight were represented in each treatment group. The average tumor size of first passage 'advanced' tumors used in this series of experiments ranged from 84 to 180 mg per experiment at the beginning of treatment.

Tumor measurement

Two axes of the tumor (the longest axis, L, and the shortest axis, W) were measured with the aid of a Vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) × (W(mm)^2)/2.

Chemotherapeutic agents

MMPR and 6-AN were obtained from Sigma (St Louis, MO). Each of these agents was dissolved in 0.85% NaCl solution immediately before use. PALA and paclitaxel were obtained from the Department of Health, Education, and Welfare (USPHS, National Cancer Institute, Bethesda, MD). PALA was dissolved in 0.85% NaCl solution and the pH was adjusted to 7.2–7.5 with 1N NaOH before adjustment to final volume. Paclitaxel was received already solubilized in polyoxyethylated castor oil and dehydrated alcohol. Because of the known toxicity of this diluent, the paclitaxel stock was diluted, depending upon the dose to be administered, a minimum of 6-fold in saline before injection. For doses below 10 mg/kg, paclitaxel was administered i.p. in 0.1 ml/10 g of body weight. For doses above 10 mg/kg, an appropriate additional volume was administered. All other agents were administered so that the desired dose was contained in 0.1 ml/10 g of mouse body weight.

PMA was administered i.p. in a timed sequence, with PALA (100 mg/kg) administered 17 h before MMPR (150 mg/kg) + 6-AN (10 mg/kg), and paclitaxel (25 mg/kg) administered 2.5 h after MMPR + 6-AN.

Determination of chemotherapy-induced tumor regression rate

The initial size of each tumor in each treatment group was recorded prior to the initiation of treatment. Tumor size was recorded weekly during treatment and again at 4–7 days after the last course of treatment. For each experiment a single observer made all measurements in order to avoid variation in caliper measurements from individual to individual. By convention, partial tumor regression is defined as a reduction in tumor volume of 50% or greater compared to the tumor volume at the time of initiation of treatment. The partial regression (PR) rate obtained from a particular treatment is expressed as a percentage, i.e. number of partial regressions per group/total number of surviving animals per group × 100.

Statistical evaluation

Differences in the size of tumors between treatment groups were compared for statistical significance by the Student's t-test. Differences between groups with $p = 0.05$, or less, were considered significant.

Results

PMA + paclitaxel achieves superior antitumor activity than paclitaxel or PMA alone

Table 1 reports four separate experiments in first generation CD8F1 murine advanced breast tumors. Each experiment consisted of four groups, each comprised of 10 tumor-bearing animals treated with (1) saline, (2) paclitaxel alone, (3) PMA alone or (4) the combination of PMA + paclitaxel. Treatment

Table 1. PMA[a] + paclitaxel achieves superior antitumor activity than paclitaxel alone against first generation CD8F1 breast tumor transplants[b]

Experiment no. (group)	Treatment	Body weight change (%)	Dead/total	Average Tumor weight (mg)	PR/survivors
2542					
1	saline	–	9/10	–	0
2	$paclitaxel_{80}$	−3	0/10	556	0
3	PMA	−11	2/10	539	0
4	PMA + $paclitaxel_{25}$	−15	0/10	62[c]	5
2544					
1	saline	−5	5/10	4152	0
2	$paclitaxel_{80}$	−6	2/10	1140	0
3	PMA	−15	0/10	312	0
4	PMA → 2.5 h → $paclitaxel_{25}$	−20	0/10	63[c]	4
2553					
1	saline	0	3/10	5283	0
2	$paclitaxel_{80}$	−9	1/10	216	0
3	PMA	−23	0/10	222	0
4	PMA → 2.5 h → $paclitaxel_{30}$	−24	1/10	44[c]	5
3029					
1	saline	−4	0/10	2461	0
2	$paclitaxel_{80}$	−2	0/10	977	0
3	PMA	−18	0/10	512	0
4	PMA → 2.5 h → $paclitaxel_{25}$	−15	0/10	416	0

[a]PALA (100 mg/kg) administered i.p. 17 h before i.p. MMPR (150 mg/kg) + i.p. 6-AN (10 mg/kg) with taxol given i.p. 2.5 h after MMPR + 6-AN (except for exp. 2542 where PMA + paclitaxel were administered concomitantly). Subscripts = mg/kg.
[b]Experiments 2542, 2544, 2553 and 3029 with tumor weights averaging 85, 115, 84 and 120 mg, respectively, at initiation of therapy. Three courses of the indicated therapy at 10–11 day intervals. Observations at 6 days after the third course.
[c]Statistically significant ($p \leq 0.05$) compared to paclitaxel alone (80 mg/kg, group 2) or PMA alone (except for exp. 3029).

with paclitaxel alone (group 2) or PMA alone (group 3) produced statistically significant inhibition of tumor growth compared to saline-treated tumors (group 1). Paclitaxel alone produced partial tumor regressions after the first two courses, but these tumors re-grew by the third course and, thus, no tumor regressions were observed in group 2 at the time of evaluation after three courses of treatment. It should be noted that courses of treatment were administered every 10–11 days and tumor measurements were recorded at 6 days after the third course of treatment; hence, there was ample opportunity for growth of paclitaxel-resistant tumor cells. The groups receiving the combination of PMA and paclitaxel (group 4) demonstrated statistically significant tumor inhibition (except for exp. 3029) compared to paclitaxel alone or PMA alone, as well as impressive tumor regression rates (i.e. 50, 40 and 55%).

In another group of five separate experiments without either saline or paclitaxel alone treatment groups, the triple drug regimen, without (group 1) or with paclitaxel (group 2), was administered at 10–11 day intervals and pooled observations were recorded 6 days after the third course of treatment.

Pooling the toxicity data of the five groups receiving only the PMA drug regimen, there was an average weight loss of 16% and a mortality rate of 12%. There were no mortalities in the PMA + paclitaxel-containing drug combination, group 2, and the weight loss (18%) was essentially identical to its PMA-treated control without paclitaxel, group 1, but the tumor-regressing therapeutic activity was significantly better ($p < 0.001$) than that achieved by the PMA drug combination without paclitaxel. It should be noted that, whereas weight loss can inhibit tumor growth, tumor regressions are never produced by weight loss. It also should be noted that the superior antitumor activity of the PMA + paclitaxel combination was achieved with a dose of paclitaxel (25 mg/kg) that was less than one-third that of the MTD of paclitaxel alone (80 mg/kg) and that this increase in antitumor activity was achieved without mortality. See Table 2.

Discussion

Importantly, the tumor-regressing effects of paclitaxel that were significantly enhanced by the prior

Table 2. Enhanced activity of paclitaxel when administered following PMA[a] ATP-depleting chemotherapy in the treatment of advanced first passage CD8F1 murine breast tumors[b]

Treatment	Body weight change (%)	Dead/total	PR/survivors
1 PMA	−16	6/49 (12%)	2/43 (5%)
2 PMA → 2.5 h → $taxol_{25}$	−18	0/48 (0%)	27/48 (56%)[c]

[a]PALA administered i.p. 17 h before i.p. MMPR (150 mg/kg) + 6-AN (10 mg/kg) and paclitaxel (25 mg/kg) administered i.p. 2.5 h after MMPR + 6-AN. Subscript = mg/kg. Observations recorded 6 days after the third course of treatment.
[b]Pooled results: exps 2659F, 2662F, 2670F, 2674F and 2675F with initial tumor weights averaging 180, 172, 130, 155 and 122 mg, respectively.
[c]Statistical significance of PR rate between group 1 and 2: $p < 0.001$.

administration of PMA were obtained with one-third the MTD of paclitaxel as a single agent. A potential benefit of the combination treatment, in addition to enhanced clinical responses over that observed with paclitaxel alone in human breast cancer, is that the ability to employ lower effective doses of paclitaxel may lower clinical toxicity associated with this agent. The toxic side effects of paclitaxel are dose-dependent and its diluent vehicle, Cremopher El, may be responsible for hypersensitivity reactions;[1] thus, a lower clinical dose of paclitaxel employed with PMA may reduce toxicities even while the antitumor effects are increased. High doses of paclitaxel have not been established to be superior to low doses in the clinic and, even if the toxicities and antitumor activity are equivalent, the lower dose will be less expensive.[1] Further, *in vivo* studies have demonstrated that, at low levels, paclitaxel blocks mitosis and inhibits cell proliferation by stabilizing spindle microtubules, apparently by a subtle effect on the inhibition of cell proliferation from the binding of small numbers of paclitaxel molecules per microtubule, and not by changing the mass of polymerized tubulin.[21–24] Importantly, low levels of paclitaxel *per se* are reported to lead not only to inhibition of cell proliferation but also to cell killing.[21]

ATP depletion appears to be of relevance in both paclitaxel-induced and DNA-damaging agent-induced apoptosis. For example, while the primary direct effect of paclitaxel is indeed directed at the microtubules, there also are 'ladder pattern' DNA breaks formed during paclitaxel-induced apoptosis[24,25] that may activate poly(ADP-ribose) polymerase (PARP), the nuclear enzyme that, when activated, depletes ATP.[26] Like paclitaxel, corticosteroids are not direct DNA-damaging agents, but nevertheless induce DNA strand breaks, followed by PARP activation, NAD consumption and ATP depletion, which precede apoptotic cell death in susceptible cancer cells.[27] This is a documented example[27] that the cytotoxicity of *non*-direct DNA-damaging agents may be mediated by DNA strand breaks that in turn induce ATP depletion. There are a number of findings common to the apoptosis induced by both types of agent. For example, p53 is an important determinant of apoptosis induction,[28] and both DNA-damaging agents[29] and paclitaxel[30] increase p53 levels, both activate p53-dependent cyclin kinases such as $p21^{WAF1/CIP1}$ and $p34^{cdc2}$,[30] and both enhance tubulin polymerization in apoptosis.[31]

The significantly enhanced tumor regression rate of PMA + paclitaxel, obtained *in vivo* in well-advanced murine solid tumors, is clearly dramatic and appears important. Therefore, while the hypothesized role of ATP depletion in the therapeutic synergism between PMA and paclitaxel is investigated, a phase I clinical trial of PMA will be conducted. The optimization of a PALA + MMPR dose and schedule at the clinical level has been previously established,[32] and only the addition of an appropriate dose of 6-AN need be ascertained in the projected PMA phase I clinical study, for phase I (and II) clinical studies of PMA + paclitaxel to be investigated in proven paclitaxel-susceptible human tumors such as breast, ovarian and esophageal cancers.

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