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Enhanced antitumor activity of an adriamycin + 5-fluorouracil combination when preceded by biochemical modulation

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A three-drug combination, PMA, consisting of (phosphonacetyl)-L-aspartic acid + 6-methylmercaptopurine riboside + 5-aminonicotinamide, preceding either 5-fluorouracil (5-FU) or adriamycin (Adr), produced tumor-regressing activity in a murine advanced breast tumor model not attainable with either 5-FU or Adr as single agents, or with any lesser combination of these drugs administered at maximally tolerated doses. Marked tumor-regressing activity was further increased significantly by using 5-FU and Adr together in conjunction with the modulatory biochemical conditioning (particularly ATP depletion) provided by pretreatment with PMA.

Key words: Adriamycin, 5-fluorouracil, modulation.

Introduction

A three-drug combination, *N*-phosphonacetyl-L-aspartic acid (PALA) + 6-methylmercaptopurine riboside (MMPR) + 6-aminonicotinamide (6-AN), or PMA, produces a mutually reinforcing block of pyrimidine and purine *de novo* biosynthesis, as well as a specific attack on nicotinamide adenine dinucleotide (NAD^+) metabolism, that summates in a damaging depletion of ATP and tumor-regressing activity, and has been described previously.[1–4] The DNA-damaging effects of anticancer agents results in a chain reaction beginning with the activation of poly (ADP-ribose) polymerase which consumes NAD pools, which in turn results in ATP depletion and consequent cell death.[5–16] The ATP-depleting effects in tumors of PMA administered prior to 5-fluorouracil (5-FU) or adriamycin (Adr), or both, would be expected to further modulate (i.e. further decrease) the ATP-depleting effects in tumors produced by the latter anticancer agents and further result in an enhancement of tumor regressing activity. Using a murine model of spontaneous breast cancer,[16,17] we have shown that the combination of PMA with either 5-FU,[4] or Adr,[3] or phenylalanine mustard[2] or radiotherapy,[2] produces a magnitude of tumor-regressing activity that cannot be attained with any of the drugs alone or with any lesser combination of agents administered at maximally tolerated doses.

5-FU and Adr often are used successfully together in the treatment of clinical breast cancer (e.g. cytoxan + Adr + 5-FU, or CAF). Therefore, these two cytotoxic drugs were combined in conjunction with PMA and the therapeutic results obtained in $CD8F_1$ mice bearing advanced first passage spontaneous breast tumors are presented in this report.

Materials and methods

Murine breast tumor system: first passage $CD8F_1$ mammary carcinoma

$CD8F_1$ mice bearing single spontaneous autochthonous breast tumors were selected from our colony, which has been described previously.[17,18] These tumors have a 100% positive chemotherapeutic correlation with human breast cancer[19] and were employed to provide first generation transplants to syngeneic 3 month old mice. This murine tumor model was included in the murine tumor testing panel of the National Cancer Drug Screening Program.[20]

As in all spontaneous tumors, whether human or murine, each individual cancer has a heterogeneous neoplastic cell population. For each experiment, the first generation transplants of $CD8F_1$ breast tumors were obtained from a tumor cell brei made by pooling three or four spontaneously-arising tumors and,

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hence, the individual transplants in each experiment develop from a single brei that, although common to all the mice in that experiment, has a neoplastic cell composition that is likely somewhat different from that in another experiment. This makes for quantitative differences between experiments. For example, a single spontaneous tumor may have a large subset of cells resistant to a particular agent and the pooling of this tumor with three more sensitive spontaneous tumors to make up a single tumor cell brei then results in the spread of these resistant cells throughout all the individual tumor transplants of the experiment. Consequently, the tumor-bearing mice in such an experiment are more refractory to tumor regressing chemotherapy than an experiment made from a tumor brei coming from three or four tumors all of which contain a paucity of resistant cells. Indeed, for this reason, an experiment comprised only of spontaneous tumors may yield therapeutic results that are more impressive than an experiment with first passage spontaneous tumors. The need to employ first passage spontaneous tumors is conditioned by economics as a tumor brei from three or four spontaneous tumors may yield 100–150 tumor-bearing animals only one transplant step away from the heterogeneity of spontaneous tumors. Quantitative measurements of any individual parameter (e.g. magnitude of sensitivity to a particular drug treatment) may be somewhat different from experiment to experiment with first passage spontaneous tumors, but the findings will be quantitatively relevant within individual experiments, as will similar trends among experiments.

In approximately 3–4 weeks, when transplanted tumors were well-advanced and measurable, the tumor-bearing mice were distributed among experimental groups of 10 mice each so that mice carrying tumors of approximately equal weight were represented in each treatment group. The mean tumor weight in each of six separate experiments ranged from 100 to 170 mg at the initiation of treatment.

Tumor measurements

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, 6-AN and 5-FU were obtained from Sigma (St Louis, MO). Adr was obtained from Adria Laboratories (Columbus, OH). Each of these agents was dissolved in 0.85% NaCl solution immediately before use. PALA was 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 1 N NaOH before adjustment to final volume. All agents were administered so that the desired dose was contained in 0.1 ml/10 g of mouse body weight. Adr was administered i.v. and the other agents i.p.

Drugs were administered in a timed sequence, with PALA at 100 mg/kg administered 17 h before the simultaneous administration of MMPR at 150 mg/kg + 6-AN at 10 mg/kg, and with 5-FU at 75 mg/kg + Adr at 6 mg/kg administered 2.5 h after MMPR + 6-AN. The latter doses of 5-FU and Adr are the MTD of each agent when given in conjunction with PAM. The MTDs for single-agent controls for each of these drugs have been previously determined empirically and have been previously published.[2–4] Three courses of the indicated treatment were administered with a 10–11 day interval between courses. Observations of antitumor activity and toxicity are reported 7 days after the third course of treatment (i.e. approximately 1 month after initiation of treatment).

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 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 with the tumor volume at the time of initiation of treatment. The partial tumor regression rate obtained from a particular treatment is expressed as a percentage, i.e. number of partial regressions per group/total number of animals per group × 100.

Statistical evaluation

Differences in the number of partial tumor regressions between treatment groups were compared for statistical significance by χ^2 analysis. Differences between groups with $p \leqslant 0.05$ were considered to be significant.

Results

Host toxicity (Table 1)

Treatment with each of the four regimens produced a similar level of acceptable toxicity with mortality rates ranging from 3% in mice treated with the five-drug regimen, PMA + Adr (group 4), to 12% in mice treated with the four-drug regimen, PMA + 5-FU (group 2) (see Table 1). The body weight loss (−17%, group 1; −19%, group 2; −25%, group 3; −25%, group 4) was not accompanied by diarrhea or by clear histopathologic changes in organs (e.g. intestine), but was clearly due to a documented severe decrease in eating and drinking for 3–4 days after each course of chemotherapy. Treatment-conditioned weight loss due to failure to eat or drink is usual for animals receiving intensive chemotherapy, and has been found by other investigators (Teicher and Frei, unpublished studies). Weight loss, which can indeed cause inhibition of tumor growth, does not produce tumor regression (Martin and Stolfi, unpublished studies). This fact is apparent in the present findings. Groups 1 and 2 have similar weight loss (−17 and −19%), but significantly different tumor regression rates (2 versus 60%, respectively). Groups 3 and 4 have identical weight loss (−25%), but significantly different tumor regression rates (60 versus 79%, respectively).

Therapeutic results

The pooled results of the therapeutic activities of these regimens in six separate experiments are reported in Table 1. In tumor-bearing mice treated with PMA (group 1), the number of regressions totaled only 1 PR (partial tumor regression, i.e. 2%) at the end of the observation period (approximately 1 month after initiation of therapy).

In group 2 (PMA + 5-FU), the number of tumor regressions totaled 30 PR plus 1 CR (complete tumor regression), a 60% objective response rate.

In group 3 (PMA + Adr), the tumor regressions, totaled 33 PR plus 1 CR in the 57 surviving mice (i.e. 60%).

In tumor-bearing mice treated with the total combination, PMA + 5-FU + Adr (group 4), tumor regressions, totaled 41 PR plus 5 CR in the 58 surviving mice (i.e. 79%). The magnitude of the tumor regression rate in group 4 (PMA + 5-FU + Adr) was found to be statistically significant when compared with group 2 (PMA + 5-FU) or to group 3 (PMA + Adr).

In therapy experiments comparing the double combination of 5-FU + Adr to the five-drug PMA + 5-FU + Adr combination, various dosage ratios of 5-FU + Adr double combination produced either only tumor growth inhibition or a markedly inferior rate of tumor regression compared with the PMA + 5-FU + Adr combination, even when high doses of 5-FU and Adr (doses producing excessive mortality) were employed as a double combination. An inferior tumor regression rate was observed in

Table 1. Enhanced therapeutic activity of 5-FU plus Adr in conjunction with PALA + MMPR + 6-AN in $CD8F_1$ mice bearing advanced first passage breast tumors[a]

Treatment[b]	Regressions/survivors[c]	Dead/total
1. $PALA_{100} \xrightarrow{17h} MMPR_{150}+6\text{-}AN_{10}$	1 PR/45 = 2%	5/50 = 10%
2. $PALA_{100} \xrightarrow{17h} MMPR_{150}+6\text{-}AN_{10} \xrightarrow{2.5h} FU_{15}$	30 PR + 1 CR/52 = 60%	7/59 = 12%
3. $PALA_{100} \xrightarrow{17h} MMPR_{150}+6\text{-}AN_{10} \xrightarrow{2.5h} Adr_6$	33 PR + 1 CR/57 = 60%	3/6 = 5%
4. $PALA_{100} \xrightarrow{17h} MMPR_{150}+6\text{-}AN_{10} \xrightarrow{2.5h} 5\text{-}FU_{75}+Adr_6$	41 PR + 5 CR/58 = 79%	2/60 = 3%

[a]Pooled results: experiments 2718F, 2777F, 2796F, 2813F, 2815M, 2816M. The mean tumor weight in each of the six separate experiments ranged from 100 to 170 mg at the initiation of treatment.
[b]Three courses of the indicated treatment with a 10–11 day interval between courses. Observations 7 days after the third course of treatment (i.e. approximately 1 month after initiation of treatment). Subscripts = mg/kg.
[c]Tumor regressions were characterized as CR (complete regression), i.e. complete disappearance of detectable tumor, or PR (partial regression), i.e. reduction in tumor size of 50% or greater, but falling short of complete disappearance, when compared with tumor size at the time of initiation of treatment.

comparison with the five-drug treatment; data not presented.

Discussion

The combination of PMA + 5-FU requires a dose of 5-FU (75 mg/kg) substantially lower than the MTD dose of 5-FU tolerated on the same schedule as a single agent (150 mg/kg) in order to avoid an unacceptable mortality rate. Similarly, it is necessary to use a dose of Adr (6 mg/kg) in the PMA + Adr combination which is substantially lower than that tolerated when Adr is administered in the same schedule as a single agent (11 mg/kg). The need to lower the doses of drugs when they are used in combination is usual and in many instances, because of the necessity to reduce doses, combinations of drugs do not prove to be therapeutically superior to the individual agents at MTD doses. Nevertheless, the therapeutic results with each of the four-drug combinations (i.e. PMA + 5-FU or PMA + Adr) proved to be significantly superior to results obtained with any lesser combination, or individual treatment with the constituent drugs at MTD doses.[3,4]

It is therefore of considerable interest that, when both 5-FU and Adr were used together in conjunction with PMA, it was not necessary to reduce the doses of these drugs beyond that which was necessary when each drug was combined individually with PMA. Therefore, 5-FU was administered at 75 mg/kg together with Adr at 6 mg/kg in conjunction with PMA without an increase in mortality beyond that obtained with either PMA + 5-FU at 75 mg/kg or with PMA + Adr at 6 mg/kg. In fact, toxicity was not increased by administering the combination of 5-FU + Adr following PMA beyond that of PMA alone; nevertheless, there was a statistically significant increase in the number of tumor regressions. Therefore, this result indicates that the selectivity of 5-FU + Adr was enhanced by the modulating activity of PMA. The increased antitumor activity without an increase in host mortality suggests that the cytotoxic activity of 5-FU + Adr + PMA was focused in tumor cells but not in normal tissues.

Nearly 11% (five of the 46 tumor regressions) of the tumor regressions following treatment with the five-drug combination (PMA + 5-FU + Adr) were complete regressions as opposed to partial regressions. Although the difference in the number of complete regressions among the treatment groups in Table 1 did not reach statistical significance, this trend seems meaningful since it is difficult to achieve a complete regression in this tumor with any drug or a combination of drugs.

The enhanced rate of tumor regressions in the overall combination is accomplished with a dose (6 mg/kg) of Adr that is almost half the MTD (11 mg/kg) of Adr as a single agent in the tumor-bearing mice. Should these findings translate to the clinical level, PMA modulation of Adr may allow for a longer and safer treatment period since a lower cumulative dose of Adr would be utilized and it is known that the cardiotoxicity induced by Adr correlates with its cumulative dose.

PMA appears to provide modulatory biochemical conditioning favorable to the therapeutic activity of 5-FU and Adr, and we have hypothesized that this modulation is associated with the ATP-depleting effect of PMA treatment. Measurements of ATP concentrations in tumors following treatment with PMA, or with PMA + 5-FU, or Adr, demonstrated a positive correlation between the magnitude of ATP depletion and the number of chemotherapeutically induced tumor regressions.[21] Similar correlative findings between enhanced depletion of ATP and enhanced tumor regressions were also found among the treated groups reported here. Thus, the ATP level in tumors from mice treated with PMA + 5-FU + Adr was significantly lower than that in tumors from mice treated with either PMA + 5-FU or PMA + Adr. This data is not presented in detail here because these correlations in themselves do not prove that PMA modulates the activity of 5-FU and Adr through depletion of ATP. The correlative findings could be a 'bystander' effect. Thus, whether or not energy-depleting strategies are the cause of the enhanced therapeutic results presented here requires further study.

Conclusion

The murine spontaneous breast cancer model employed in these therapeutic studies is the only preclinical tumor model with a published 100% positive chemotheapeutic correlation with human breast cancer. The therapeutic findings are evaluated using the clinical endpoint of tumor regressions under conditions (i.e. multiple courses of *in vivo* therapy) that appear translatable to the treatment of human cancer. These results suggest a high priority for translation into the clinical setting.

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