Biochemical Modulation of Tumor Cell Energy: Regression of Advanced Spontaneous Murine Breast Tumors with a 5-Fluorouracil-containing Drug Combination

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ABSTRACT

This report describes a highly active chemotherapeutic drug combination, consisting of N-(phosphonomethyl)-L-aspartate plus 6-mercaptopurine riboside plus 6-aminonicotinamide plus 7-fluorouracil, in CD8F1 mice bearing spontaneous, autochthonous, breast tumors or first-passage advanced transplants of these spontaneous tumors. The combination and sequence of administration of these drugs were selected on the basis of known potentiating biochemical interactions. High performance liquid chromatography and nuclear magnetic resonance spectroscopy measurements of biochemical changes resulting from treatment with N-(phosphonomethyl)-L-aspartate plus 6-mercaptopurine riboside plus 6-aminonicotinamide indicated a severe depletion of cellular energy levels in the treated tumors. 6-Aminonicotinamide produced a severe block of the pentose shunt, and 7-fluorouracil severely inhibited both thymidylate synthase and thymidine kinase in the treated tumors. This quadruple drug combination, administered on a 10-11-day schedule, produced an impressive partial tumor regression rate of 67% of large, spontaneous, autochthonous, murine breast tumors and a tumor regression rate of 74% of first-passage transplants of the spontaneous breast tumors.

INTRODUCTION

ATP is the key energy source in major metabolic processes such as biosynthesis, active transport, and DNA repair. Consequently, if ATP production is inhibited, consumption of existing ATP will result in an energy deficiency that would adversely affect the functional and morphological integrity of the cell. Selective depletion of ATP (i.e., preferential depletion) in cancer cells can, therefore, be envisioned as an approach for the chemotherapy of cancer.

Although glycolysis provides some of the ATP needed by the cell, the major generation of ATP occurs during oxidative phosphorylation in the mitochondria of mammalian cells, as electrons are transferred from NADH to O2 by a series of electron carriers. NADH is reconverted to NAD+, with concomitant conversion of ADP to ATP. In addition to their central role in energy metabolism, NAD+ and NADP+ are oxidizing agents which function as coenzymes in critical biochemical reactions. Disruptions in NAD+ synthesis and metabolism could have profound adverse effects on cellular integrity, because of the central role played by these coenzymes in intermediary metabolism, including the generation of ATP from ADP. Limitation of adenine or NAD+, or both, is the key to ATP depletion.

These metabolites are, therefore, targets for chemotherapy designed to result in depletion of cellular levels of ATP. Of available chemotherapeutic drugs which might be useful for this purpose, we have examined the nicotinamide antagonist (1) 6-AN3 and the adenosine analogue MMPR.

6-AN is converted in vivo into the NAD+ analogues 6-AN adenine dinucleotide and 6-AN adenine dinucleotide phosphate. These competitive analogues of NAD+ and NADP+ cannot be reduced either chemically or enzymatically (2-4) and, consequently, act as potent inhibitors of NAD-dependent dehydrogenases utilized in glycolysis, in the oxidative portion of the pentose phosphate pathway, and in mitochondrial oxidative phosphorylation (2-12). 6-AN has demonstrated preclinical anticancer activity (13, 14).

MMPR has been shown to result in ATP and GTP depletion, inhibition of macromolecular synthesis, and inhibition of tumor growth (15-20). In addition, the biosynthesis of NAD+ may be inhibited by MMPR, because NAD+ is synthesized in the cell from nicotinamide mononucleotide and ATP, by an enzyme (nicotinamide mononucleotide adenyl transferase) that is inhibited by thio-inosine triphosphate (21). Theoretically, the combination of MMPR and 6-AN would be expected to be synergistic if the timing of their administration was appropriate, because the lowering of NAD+ levels by MMPR should favor the competition of 6-AN adenine dinucleotide with NAD+ and thereby enhance the magnitude of ATP depletion that could be achieved by either drug alone. We have recently reported that the combination of 6-AN and MMPR can produce regressions of advanced murine breast tumors which cannot be obtained with either drug alone (22). In addition to its antipurine action, MMPR, in high dosage, has been reported to decrease pyrimidine ribonucleotide concentrations in vivo (18, 23). Because the pyrimidine antagonist PALA, in low nontoxic dosage, can lower pyrimidine levels selectively in certain tumors in vivo (24), it seemed reasonable that PALA might provide a worthwhile addition to the 6-AN plus high-dose MMPR therapy, because of its ability to reinforce the inhibition of pyrimidine synthesis.

On the basis of the rationale outlined above, the triple combination of PALA plus MMPR plus 6-AN was evaluated against advanced solid tumors in mice (25). Preliminary biochemical data (26) demonstrated substantial depression, at 24 and 48 h, of levels of NAD+ and of the four ribonucleoside triphosphates of purines and pyrimidines, including ATP. The three-drug combination produced a marked antitumor effect, which could not be obtained with any of the individual agents or any combination of two of the agents (22, 25).

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3 The abbreviations used are: 6-AN, 6-aminonicotinamide; MMPR, 6-mercaptopurine riboside; PALA, N-(phosphonomethyl)-L-aspartate; FURA, 5-fluorouracil; NTP, nucleoside triphosphate; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; PRPP, 5-phosphoribosyl pyrophosphate; GSH, reduced glutathione; GSSG, oxidized glutathione.
The PALA/MMPR/6-AN-induced reduction in all four individual ribonucleoside pools (26), which generally correlates with a reduction in the corresponding deoxyribonucleoside triphosphate pools (27), would be expected not only to deplete cellular energy sources and to inhibit DNA synthesis but also to inhibit the potential for DNA repair. Because the DNA damage produced by many chemotherapeutic drugs is subject to repair, the cytotoxic activity of these DNA-damaging drugs may be expected to be increased when the DNA repair potential of the cell is decreased. Therefore, we hypothesized that the three-drug combination, PALA plus MMPR plus 6-AN, would augment the antitumor activity of DNA-damaging drugs by virtue of its ability to deplete both the deoxyribonucleotides and the energy source required for the DNA repair processes.

The underlying molecular mechanism of fluoropyrimidine-induced "thymineless death" has been shown to be due to "programmed cell death" (apoptosis) activated by DNA strand breakage (28). Measurements of biochemical changes in CDF8F1 breast tumors after in vivo administration of the PALA/MMPR/6-AN drug combination reveal severe ATP loss, inhibition of macromolecular synthesis, inhibition of the pentose phosphate shunt, depletion of NAD, reduction of ribonucleoside triphosphates, and inhibition of protein synthesis, a pattern of findings which overlap with those reported in thymineless death and apoptosis. Thus, the similarity between the biochemical findings induced by our triple combination and those found in programmed cell death suggested that the addition of an apoptosis-inducing anticancer agent, such as FUra (28, 29), to the triple combination would result in complementary therapeutic activity.

Also, in view of the expected PALA/MMPR/6-AN-induced elevation of PRPP levels and reduction of UTP pools, it seemed likely that the triple drug combination would also increase the therapeutic activity of FUra, by increasing the anabolism of FUra to its nucleotides as well as by favoring the competition of the analogue over the natural pyrimidine intermediates, whose levels had been reduced by PALA. Our previous experience with two of the drugs, PALA and MMPR, in effectively augmenting the activity of FUra in preclinical studies (24-26) has resulted in successful clinical trial of one drug sequence (i.e., PALA plus FUra) that produces this biochemical modulation (30, 31).

This study reports an impressive increase in tumor regression rates with FUra administered in conjunction with PALA, MMPR, and 6-AN, in the therapy of either advanced first-passage or spontaneous murine breast tumors, and the results of measurements of biochemical parameters affected by treatment.

MATERIALS AND METHODS

Murine Breast Tumor System

Spontaneous, Autochthonous, Mammary Carcinoma. CDF8F1 hybrid mice bearing single, spontaneous, autochthonous, breast tumors arising during the preceding week were selected from our colony, which has been described previously (32, 33). All tumors were measured with calipers, and the mice were distributed among experimental groups so that mice carrying tumors of approximately equal weight were represented in each treatment group. Individual tumors ranged in size from 100 to 500 mg, and the average tumor weight in all groups was 260 mg at the beginning of treatment.

First-Passage CDF8F1 Mammary Carcinoma. For each experiment, the spontaneously arising CDF8F1 breast tumors were transplanted into syngeneic 3-month-old mice. As in all spontaneous tumors, whether human or murine, each individual cancer has a heterogeneous neoplastic cell population. The first-generation transplants of CDF8F1 breast tumors were obtained from a tumor cell brei made by pooling three or four spontaneously arising tumors. Thus, the individual transplants in each experiment developed from a single brei that, although common to all the mice in that experiment, had a neoplastic cell composition that was likely slightly different from those in other experiments. Therefore, quantitative measurements of any individual parameter (e.g., thymidylate synthase activity or average tumor size) may be somewhat different from experiment to experiment, but the findings are quantitatively relevant within individual experiments, as are similar trends among experiments. The CDF8F1, first-generation breast tumor is included in the murine tumor testing panel of the National Cancer Drug Screening Program (34).

In approximately 3-4 weeks, when transplanted tumors were measurable, the tumor-bearing mice were distributed among experimental groups so that mice carrying tumors of approximately equal weight were represented in each treatment group. The average tumor weight was close to 125 mg at the beginning 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 as

\[
\text{Tumor wt (mg)} = L \text{(mm)} \times \frac{W \text{(mm)}^2}{2}
\]

Chemotherapeutic Agents

MMPR, 6-AN, and FUra were obtained from Sigma Chemical Co. (St. Louis, MO). 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, from the 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 i.p., with the desired dose contained in 0.1 ml/10 g of mouse body weight.

These drugs were administered in a timed sequence, with PALA being administered 17 h before the simultaneous administration of MMPR plus 6-AN and 5-FUra being administered 2.5 h after MMPR plus 6-AN. Throughout this paper, the timing of biochemical measurements is given in relation to the injection of MMPR plus 6-AN in the chemotherapeutic sequence.

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 7-9 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 more, compared to the tumor volume at the time of initiation of treatment. The partial 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 x² analysis. Student's t test was used for evaluation of measured biochemical differences between treatment groups. Differences between groups with \( P \leq 0.05 \) were considered to be significant.

Incorporation of Precursors into RNA and DNA

The radiolabeled precursors \((^{32P})H_2PO_4 \) and \( l-[^{3}H] \)leucine were administered i.p.; the labeling period was 2 h. At the end of the labeling...
period, animals were sacrificed by cervical dislocation. Tumor tissues were homogenized in TNE buffer (0.01 M Tris-HCl, pH 7.6, 0.15 M NaCl, 0.001 M EDTA) containing 1% Triton X-100. The homogenate was briefly sonicated and then digested with Pronase (0.2 mg/ml) for 60 min at 37°C; finally, this material was extracted with chloroform-isooamyl alcohol (24:1, v/v). Samples of extracted material were precipitated with trichloroacetic acid for determination of total radioactivity. Other samples were first treated with alkali (0.4 M NaOH, for 90 min at 37°C) for determination of alkali-stable, trichloroacetic acid-pellicable radioactivity. The difference between the total and alkali-stable radioactivity was assumed to represent radioactivity in RNA. 5-6-H]-Fluorouracil (20 Ci/mmol), [methyl-3H]-thymidine (60 Ci/mmol), and [5-3H]deoxyuridine monophosphate (20 Ci/mmol) were purchased from Moravek.

Substrate Accumulations

Cellular levels of 6-phosphogluconate, glucose-6-phosphate, and fructose-6-phosphate were measured in perchloric acid extracts by published methods (6-phosphogluconate, Ref. 35; glucose-6-phosphate and fructose-6-phosphate, Ref. 36).

[14C]Orotic Acid Assay for PRP

The assay was based on the conversion of [14C]orotic acid to UMP, with release of [14CO2], by orotidine-5-phosphate-pyrophosphorylase plus orotidine-5-phosphate decarboxylase (37). An aliquot of the homogenate was assayed for protein content by the method of Lowry et al. (38).

Processing of Samples for NTP Content

Frozen tumor specimens were homogenized in ice-cold 1.2 n perchloric acid. The acid-insoluble fraction was removed by centrifugation (7000 rpm for 15 min). The acid-soluble fraction was neutralized by extraction with a mixture of Freon and tri-n-octylamine (2:1). The extract was then filtered through a 0.22-μm Millipore membrane filter prior to HPLC analysis. NTP contents in tumors were normalized to the protein content of the acid-insoluble fraction.

Measurements of NTP Levels in Tumors

HPLC analysis of tumors was performed on a Waters 840 HPLC system with a WISP automatic sampler. NTP levels were analyzed by ion-exchange gradient chromatography, using a Waters SAX column, starting with 3 mm NH4H2PO4, pH 3.5, and proceeding in two steps to 70% 0.5 m NH4H2PO4, pH 5.0/30% starting buffer. The run time for each 100 μl of extracted sample was 60 min. Tumor NTP levels are expressed as micrograms of nucleotide triphosphate per milligram of protein.

31P NMR Spectra

31P NMR spectra were obtained using techniques described previously (39). Briefly, spectra were obtained with a General Electric NT-300 wide-bore spectrometer operating at 121.5 MHz. Experimental parameters included a spectral width of ±12,000 Hz, 60° tip angle, recycle delay of 2 s, 512–1024 averaged free induction decays, and 1024 data points. The spectra are partially saturated using these experimental conditions. Four-turn solenoid coils, with a Faraday shield (40) positioned between the body of the mouse and the coil, were used to detect the NMR signal. Control experiments verified that no signal was obtained from non-tumor-bearing animals mounted in an identical manner. Spectra were analyzed using 25-Hz exponential multiplication, followed by Fourier transformation. Spectral peak areas were estimated by fitting the spectra to a series of Lorentzian peaks, using a program (GEMCAP) available on the spectrometer, after fitting the base line to a third-order polynomial (using standard General Electric software). Since other peaks overlap the α and γ NTP peaks, the β NTP peak was used for calculation of NTP peak area ratios.

Thymidine Kinase and Thymidylate Synthase Assays

Tissues were homogenized (Potter-Elvehjem homogenizer) as a 20% (w/v) solution in Tris-HCl (100 mm, pH 7.6), with 2-mercaptoethanol (20 mm) and sodium fluoride (100 mm). Homogenates were centrifuged (100,000 × g for 60 min or 10,000 × g for 30 min), and the supernatant fractions were retained on ice. Enzyme assays were performed either on individual samples or on pooled tissues from three animals. Tumors were 300–500 mg. Thymidine kinase was measured immediately after cytosol preparation, by means of a DE81 filter-binding assay (41). The assay mixture contained Tris-HCl (100 mm, pH 7.6), ATP (5 mm), MgCl2, [5-'H]thymidine (25 μM, 1.0 Ci/mmole), and cytosolic protein. Thymidylate synthase activity was measured as the release of tritium from [5-'H]dUMP (10 μM, 1.0 Ci/mmole), with CH2H4PteGlu (100 μM), in the cytosolic fraction (25 μl) (42). Reactions were terminated by the addition of perchloric acid (10 μl, 0.7 n). Protein was determined by the method of Lowry et al. (38).

Incorporation of FUra into RNA

Incorporation of FUra into RNA was determined by isolating tumor RNA by the acid-guanidinium isothiocyanate procedure (43), after treatment with [5-3H]FUra (2.0 μCi/mmol). Tissues were harvested 4–5 h after treatment, immediately frozen in liquid nitrogen, and stored at –70°C. Tissues were homogenized (80 mg/ml) in the denaturing solution (citrate-sarcosyl laurate-2 mercaptoethanol) and extracted with 1 volume of phenol-chloroform-isooamyl alcohol (50:40:2). After extraction, the RNA was precipitated with alcohol, washed, redissolved, quantified by UV, and then precipitated with perchloric acid. This final precipitate was collected on GF-C filters, and the radioactivity was determined.

RESULTS

Chemotherapeutic Effects on Breast Tumor Transplants

In a series of 5 similar experiments, a group (group 1) of CDF1 mice bearing advanced, first-passage, spontaneous CDF1 breast tumor transplants were treated with the triple combination of PALA plus MMPR plus 6-AN (Table 1). PALA (100 mg/kg) was administered 17 h before MMPR (150 mg/kg) plus 6-AN (10 mg/kg). A second group (group 2) received the same treatment with PALA plus MMPR plus 6-AN, followed 2.5 h later by FUra (75 mg/kg). Treatment was repeated at 10- or 11-day intervals for a total of 3 courses, and observations were recorded 7 days after the last course of treatment.

Partial tumor regressions were observed in 8 of the surviving 48 mice (17%) treated with PALA plus MMPR plus 6-AN (group 1). The range of regression rates in individual experiments varied from 0 to 30%. The addition of FUra to the same regimen of PALA plus MMPR plus 6-AN (group 2) produced

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Body weight change (%)</th>
<th>Dead/total</th>
<th>Partial regressions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PALA100</td>
<td>−16</td>
<td>1/49 (2%)</td>
<td>8 (17%)</td>
</tr>
<tr>
<td></td>
<td>MMPR150</td>
<td>+ 6-AN10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PALA100</td>
<td>−22</td>
<td>0/50 (0%)</td>
<td>37 (74%)</td>
</tr>
<tr>
<td></td>
<td>MMPR150</td>
<td>+ 6-AN10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.5 h) FUra</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Three courses of the indicated treatment were administered, with a 10- or 11-day interval between courses. Subscripts refer to doses, in mg/kg. Observations were recorded 7 days after the third course of treatment.
a significant and meaningful increase in therapeutic activity. Thirty-seven of the 50 treated mice (i.e., 74%) experienced partial tumor regressions (with a range in the individual experiments of 60–90%). This level of therapeutic activity was found to be significantly better than that achieved with the three-drug combination (without FUra, group 1; P < 0.001) and, most importantly, this increase in antitumor activity was achieved without mortality. It should be noted that FUra alone at 75 mg/kg did not produce any tumor regressions after 3 courses of treatment, in 9 separate experiments (0 of 88 mice; data not shown).

In 3 separate experiments the therapeutic activity of the PALA plus MMPR plus 6-AN plus FUra combination was compared with that of the combination of PALA plus MMPR plus FUra (i.e., without 6-AN), at the same doses and administration schedule. One week after three courses of treatment, the four-drug combination yielded a 71% tumor regression rate (20 tumor regressions in 28 surviving mice), whereas the three-drug combination without 6-AN yielded a regression rate of only 41% (12 tumor regressions in 29 surviving mice). The difference in regression rate between these two treatments was found to be statistically significant (P < 0.05), indicating that the low-dose 6-AN was contributing to the therapeutic activity in the four-drug combination.

Chemotherapeutic Effects on Spontaneous, Autochthonous, Breast Tumors

The PALA plus MMPR plus 6-AN regimen, with or without FUra, was administered at 10–11-day intervals, in a series of 6 experiments, to large numbers (a total of 66 or 67 mice in each treatment arm) of CD8F, mice bearing advanced, spontaneous, autochthonous, breast tumors averaging 260 mg (Table 2). Results were observed at 6 weeks after the initiation of treatment (i.e., 9 days after the fourth course of treatment). The PALA plus MMPR plus 6-AN regimen (group 1) produced a partial tumor regression rate of 38% in mice bearing spontaneous, autochthonous, breast tumors (24 partial tumor regressions in 64 surviving mice), with an acceptable mortality rate (4%; 3 deaths in 67 treated mice). The addition of FUra to the three-drug regimen (group 2) resulted in 41 partial regressions in the 61 surviving mice, or 67% (with a range of 50–90% in the individual experiments), without an increase in the mortality rate (only 7%) and with only 10% body weight loss. As in the previous experiments in mice bearing first-passage tumors, the addition of FUra to the PALA/MMPR/6-AN regimen resulted in a significant increase in tumor regressions (P < 0.01) in mice bearing spontaneous, autochthonous, breast tumors. Again, it should be noted that FUra alone at 75 mg/kg produced <5% regressions of spontaneous, autochthonous, CD8F, breast tumors (1 regression in 24 treated tumors), and spontaneous regressions of these tumors has not been observed.

Biochemical Findings

Macromolecular Synthesis. Administration of PALA/MMPR/6-AN resulted in significant inhibition of macromolecular synthesis, in first-passage CD8F, breast tumors, that was detectable at the earliest time point examined, 2.5 h after drug administration, and progressed to 80% inhibition of DNA synthesis, 85% inhibition of RNA synthesis, and 70% inhibition of protein synthesis at 48 h.

Pentose Shunt and Glycolytic Intermediates. 6-AN has been reported to inhibit the pentose phosphate pathway by producing a metabolic block of 6-phosphogluconate dehydrogenase (11). The accumulation of this substrate results in feedback inhibition of phosphoglucone isomerase and prevents the formation of fructose-6-phosphate from glucose-6-phosphate (44, 45). As is evident in Table 3, administration of 6-AN did, in fact, result in significant elevation of 6-phosphogluconate (167-fold increase above saline-treated control) and glucose-6-phosphate (3-fold increase above control) in CD8F, breast tumors. Similar results were obtained in tumors from mice treated with the 6-AN-containing three-drug combination (PALA/MMPR/6-AN). We have previously reported that the three-drug combination reduces NAD+ levels in this tumor (26).

Estimation of Cellular Energy Levels by NMR. NMR spectra were obtained from first-passage CD8F, breast tumors prior to treatment and at 2, 10, and 24 h after treatment with PALA/MMPR/6-AN. Base-line spectra were similar to those obtained in previous studies (46). After treatment, a decrease in the δ NTP peak, relative to P3', was noted. The results obtained from 7 tumor-bearing animals treated in this manner are summarized in Fig. 1, which shows that both phosphocreatine/P1 and NTP/P3 ratios were decreased after treatment with this drug combination, which is indicative of energy depletion. The changes in phosphocreatine/P1 and NTP/P3 at 10 h were found to be statistically significant.

Biochemical Measurement of ATP Levels. Energy depletion in drug-treated tumors also was manifest in decreased ATP
TUMOR CELL ENERGY MODULATION

Fig. 1. Changes in phosphocreatine/Pi and NTP/Pi ratios after treatment with the triple drug combination. The changes in phosphocreatine/Pi and NTP/Pi at 10 h are statistically significant (P < 0.01 and P < 0.02, respectively) when compared to pretreatment ratios. The change in NTP/Pi between the 10-h and 24-h measurements is not significant. Pretreatment values were determined before the administration of PALA (100 mg/kg) and appear on the y-axis. At time 0, MMPR (150 mg/kg) plus 6-AN (10 mg/kg) were administered. For each time point, n = 7.

As shown in Table 4. At 6 and 24 h after administration of MMPR alone (group 2), 6-AN alone (group 3), or the three-drug combination PALA/MMPR/6-AN (group 4), ATP levels in first-passage CD8F1 tumors were significantly depressed, reaching a level of 32% of control in tumors from mice treated with the three-drug combination at 24 h after treatment.

Biochemical Changes Favoring the Activation and Competitive Activity of FUra. As a consequence of the block of de novo purine synthesis, the administration of MMPR has been shown to result in the accumulation of PRPP in CD8F1 tumor cells, and, when MMPR is administered appropriately before FUra, the increased levels of PRPP result in enhanced activation of FUra (47). Because of the possibility of complex interactions among drugs in combination, it was necessary to verify that the MMPR-containing three-drug combination also was capable of producing this elevation of PRPP in tumor cells. Accordingly, PRPP levels were measured in untreated first-passage CD8F1 tumors and in CD8F1 tumors at 3 and 24 h after treatment with PALA/MMPR/6-AN. Measurements from 4 untreated tumors in each of 3 separate experiments yielded an average PRPP level of 288 pmol/mg, with a SE of 19 pmol/mg. PRPP levels rose to 490 ± 63 pmol/mg (i.e., 2.2-fold increase; P < 0.01) and 833 ± 153 pmol/mg (i.e., 3.7-fold increase; P < 0.05) at 3 and 24 h, respectively, after administration of PALA/MMPR/6-AN.

As a consequence of the inhibition of aspartate transcarbamylase, the administration of PALA alone has been demonstrated to result in depletion of UTP pools in CD8F1 tumors, and, when PALA is administered appropriately before FUra, this results in an augmentation of the competitive activity of the analogue, FUTP (24). Again, we measured UTP levels in first-passage CD8F1 breast tumors at 24 h after administration of PALA/MMPR/6-AN and found statistically significant depression of UTP pools, compared to saline-treated control tumors (data not shown).

Therefore, two of the drugs (MMPR and PALA) in the three-drug combination produce the same biochemical alterations in CD8F1 breast tumor cells when administered in the three-drug combination as they did when administered as single agents, and these alterations have been demonstrated to result in the augmentation of subsequently administered FUra.

Biochemical Measurements of FUra Activity when Administered 2.5 h after PALA/MMPR/6-AN. Since the inhibition of RNA synthesis is one of the earliest measurable events after the administration of the triple combination, the effect of the combination upon the incorporation of FUra into RNA is of interest. The amount of tumor RNA containing FUra in the group which received PALA plus MMPR plus 6-AN plus FUra was appreciable (355 ± 127 cpm/mg RNA). Thus, although RNA synthesis was significantly inhibited following treatment with PALA/MMPR/6-AN, the incorporation of FUra into residual newly synthesized RNA was not inhibited.

Three groups of 5 CD8F1 mice bearing first-passage breast tumors of equal size were treated with saline, PALA/MMPR/6-AN, or PALA/MMPR/6-AN followed 2.5 h later by FUra. Measurements of thymidine kinase and thymidylate synthase activity 24 h after treatment are shown in Table 5. Thymidylate synthase activity was decreased by nearly 50% after treatment with PALA/MMPR/6-AN, even without the addition of FUra (group 2; Table 5). This depression of enzyme activity is likely due to the general inhibition of protein synthesis following treatment with the three-drug combination described above. However, the addition of FUra 2.5 h following treatment with the three-drug combination (group 3; Table 5) resulted in >76% inhibition of this enzyme. This increased level of inhibition is believed to be a result of 5-fluorodeoxyuridine monophosphonate inhibition of residual thymidylate synthase activity.

### Table 4 Effect of PALA, m-¿ (17 h) MMPR,8o plus 6-AN,7.1 on tumor ATP pools in CD8F1 female mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>ATP (µg/mg protein)</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>7.1 ± 0.32</td>
<td>7.1 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MMPR150</td>
<td>3.3 ± 0.32² (47%)</td>
<td>3.3 ± 0.36² (47%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6-AN10</td>
<td>5.5 ± 0.21² (77%)</td>
<td>5.3 ± 0.13³ (75%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PALA → (17 h) MMPR150 + 6-AN10</td>
<td>3.9 ± 0.11² (55%)</td>
<td>2.3 ± 0.31³ (32%)</td>
<td></td>
</tr>
</tbody>
</table>

* Subscripts refer to doses, in mg/kg body weight (i.p. injection).
* Significant; P value ≤ 0.05.
* Values in parentheses, percentage of saline control (group 1).

### Table 5 Tumor thymidine kinase and thymidylate synthase activities 24 h after treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>100.00 ± 22.30</td>
</tr>
<tr>
<td>2</td>
<td>PALA100 → (17 h) MMPR150 + 6-AN10</td>
<td>19.63 ± 4.28</td>
</tr>
<tr>
<td>3</td>
<td>PALA100 → (17 h) MMPR150 + 6-AN10 + 2.5 h FUra27</td>
<td>6.26 ± 1.61</td>
</tr>
</tbody>
</table>

* Subscripts refer to doses, in mg/kg.
* Average control activity = 46.0 pmol/min/mg protein.
* Average control activity = 12.71 pmol/min/mg protein.
We have previously reported (48) that thymidine kinase activity is lost over 24 h in CD8F, tumors from mice treated with the maximum tolerated dose (100 mg/kg) of FUra alone. The triple combination (group 2; Table 5) produced approximately 80% inhibition of thymidine kinase (apparently due to inhibition of RNA synthesis), while the addition of FUra (75 mg/kg) to the triple combination (group 3; Table 5) provided further inhibition (93.7%) of thymidine kinase activity.

DISCUSSION

The three-drug combination (PALA plus MMPR plus 6-AN) was designed to produce a mutually reinforcing blockade of pyrimidine and purine de novo biosynthesis, as well as a specific attack on NAD+ metabolism, that in toto would result primarily in a damaging depletion of high-energy nucleoside triphosphates, particularly ATP. The reasons for including the purine analogue MMPR and the nicotinamide analogue 6-AN have been presented in “Introduction,” but a few additional details might be useful regarding PALA, an inhibitor of de novo pyrimidine biosynthesis (49, 50). PALA has been found to be non-toxic to the hemopoietic systems in mice (51, 52) and, when administered at a low dose (100 mg/kg), exerts selective activity in CD8F; breast tumor tissue but not in the intestinal tissue of the host (24). Therefore, in the three-drug combination, PALA was administered at 100 mg/kg with the expectation that de novo pyrimidine biosynthesis would be inhibited selectively in breast tumor cells but not in the hemopoietic compartment or in the intestinal mucosa of the host.

In initial studies of this combination, the PALA plus MMPR plus 6-AN treatment was administered at 7-day intervals. However, more experience has demonstrated that it is less toxic when the interval between courses is extended from 7 days to 10 or 11 days and that this change in the schedule of administration reduces toxicity sufficiently to permit the safe addition of other drugs (e.g., FUra) every 10 or 11 days, together with the three-drug combination.

The PALA/MMPR/6-AN-induced reduction of ATP levels was demonstrated by both HPLC (Table 3) and the noninvasive technique of NMR spectroscopy (Fig. 1). Although the degree of suppression of ATP was significant at early time points after the triple chemotherapy, by 72 h the ATP levels had returned to normal (data not shown). In some normal cells (e.g., hepatocytes), maintenance of severely depressed levels of ATP (20% of control values) for as long as 36–48 h does not necessarily compromise viability, as long as the adenine nucleotide concentrations then return to control values (as they did in our tumor experiments) (53). Sustained or permanent loss of ATP is incompatible with cell survival (54). However, a temporary (48-h) depletion of ATP is never the sole determinant compromising cell viability. As may be appreciated by considering the known key role that ATP plays in the cell economy, any significant drop in ATP concentration has many metabolic consequences, and it is likely that other induced perturbations to cellular biochemistry (e.g., those induced by PALA and 6-AN) would act in concert with ATP loss to cause cell death (55). For example, the pentose phosphate shunt provides reducing equivalents, in the form of NADPH, for certain anabolic reactions and also for the maintenance of GSH. GSH is a major cellular reductant (56–59) and, in the process of detoxification of radical species, becomes GSSG. NADPH is required for the conversion of GSSG back to GSH, by the enzyme glutathione reductase. Thus, inhibition by 6-AN of the pentose phosphate shunt can lead to a lowering of NADPH levels, which, in turn, can prevent adequate GSH resynthesis from GSSG. And, since ATP is required for the initial synthesis of GSH from its constituent amino acids (56), the ATP depletion induced by the triple combination can limit supplies of GSH. Thus, the two effects of ATP and NADPH depletion can complement each other in this regard and, when the level of GSH falls substantially after chemical injury, cell death usually ensues (60).

The elevation of PRPP levels and the lowering of UTP levels by the triple combination were expected to facilitate the conversion of FUra into its active nucleotides for effective blockade of key FUra-sensitive enzymes, and this was, indeed, accomplished (Table 5). Thus, the addition of FUra, which at 75 mg/kg as a single agent causes few (<5%) regressions and only inhibits the growth of spontaneous CD8F breast tumors, markedly increased the tumor regression rate of the triple combination, from 38% to 67% (Table 2).

Three of these drugs (PALA, MMPR, and FUra) currently are used clinically as components of various drug combinations. A combination of PALA plus FUra has proven to be significantly more active than FUra alone in the clinical treatment of colorectal cancer (30, 31). In recent phase I clinical trials designed to determine the optimal dose and schedule of MMPR for combination with either FUra or PALA plus 5-FUra, it was clearly demonstrated that MMPR can result in the elevation of PRPP levels in human tumors such as colon, ovary, and breast (61–63); therefore, the addition of MMPR may be expected to augment the metabolic activation of FUra in human tumors, as it has been demonstrated to do in murine tumors.

6-AN was abandoned after clinical trial in the early 1960s because of a lack of efficacy as a single agent. However, at that time, the daily administration of anticancer agents was in vogue. 6-AN was administered on a daily schedule that resulted in a cumulative toxicity of nicotinamide deficiency that could be reversed merely by stopping treatment with 6-AN or by administering the antidote, nicotinamide (64). Today, the intermittent administration of anticancer drugs has proven practical utility in cancer treatment. As an intermittently administered biochemical modulator (i.e., once every 10-11 days), 6-AN should not produce signs of nicotinamide deficiency. In the present preclinical studies, a low-dose intermittent (once every 10–11 days) schedule rather than a daily administration schedule of 6-AN was employed successfully. In the early clinical trials it had been determined that a dose of 6-AN of 0.2 mg/kg/day for up to 28 consecutive days of treatment was safe (64). Using the equivalent surface area dosage conversion factor (65), this human dose was found to be equivalent to a cumulative dose of 24 mg/kg over 10 days in the mouse. Therefore, our intermittent low-dose schedule (10 mg/kg every 10–11 days) is calculated to be less than one half of the safe cumulative human equivalent dose of 6-AN every 10 days. The toxicity identified when 6-AN was administered on a daily schedule in early clinical studies should not impede the current clinical development of this agent in a therapeutic drug combination proven preclinically safe in an intermittent administration schedule.

The term biochemical modulation refers to the pharmacological manipulation of metabolic pathways by an agent (the modulating agent) to produce the selective enhancement of the antitumor effect of a second agent (the effector agent) (25). In this context, the triple combination, PALA plus MMPR plus 6-AN, may be viewed as biochemical modulation employed to establish in tumor cells a wide array of biochemical changes,
i.e., a primary diminution of ATP, lowering of all of the nucleoside triphosphates and NAD, inhibition of macromolecular synthesis, and suppression of the flux through the pentose phosphate shunt and glycolytic pathways, thereby establishing a level of disrupted metabolism in cancer cells that would complement and, indeed, be complemented by the cascade of similar biochemical derangements induced by the apoptotic effects of DNA-damaging anticancer agents (e.g., FURA, cisplatin, and bischloroethyl nitrosourea) (29, 66). This modulation would be expected to result in enhanced cancer cell death, which would be reflected, as in the results presented here, in improved tumor regression rates. Further definition of the critical apoptotic events produced by the combination of these anticancer agents should reveal additional metabolic reactions where more targeting could further enhance tumor cell cytotoxicity.

The underlying molecular mechanism of cell death following DNA damage by various chemical agents appears to consist of a uniform sequence of events, termed apoptosis. This is a programmed energy-dependent active process which is activated by DNA damage, which in turn triggers a cascade of damaging biochemical events (initially induced by NAD+ and ATP loss) that results in cell death (54, 55, 60, 66–69). The lowering of NAD+ levels and the depletion of ATP pools that have been observed in the studies of drug-induced apoptosis were also seen here in the PALA/MMPR/6-AN–treated tumor cells. Therefore, biochemical and therapeutic findings presented here support our overall rationale for combining DNA-damaging agents with the PALA/MMPR/6-AN combination and encourage the preclinical addition of other DNA-damaging agents to the triple combination. FURA is the first addition of a specific DNA-damaging agent to the PALA/MMPR/6-AN modulating combination that we are reporting, but our preliminary antitumor results with other DNA-damaging agents (e.g., Adriamycin) are also supportive of this approach, and a preliminary report of the latter findings has been presented (70).

Therapeutic activity has been measured in these studies using a stringent clinical criterion of tumor regression (i.e., 50% or greater drug-induced decrease in tumor size), rather than the more conventional animal model criteria of tumor growth inhibition. It should also be noted that the spontaneous, autochthonous, CD8βT tumor model has demonstrated a remarkable correlation with human breast cancer in terms of both positive and negative sensitivity to individual chemotherapeutic drugs, using tumor regression as the criterion for evaluation (65). Viewed against the entire background of findings, it would appear reasonable to consider this four-drug combination for clinical evaluation.

REFERENCES
Biochemical Modulation of Tumor Cell Energy: Regression of Advanced Spontaneous Murine Breast Tumors with a 5-Fluorouracil-containing Drug Combination


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