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Levels of Viral Glycoprotein Provide a Measure of Modulated Chemotherapeutic Effect

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Abstract – A chemosensitivity assay with small replicate Mm5mt/cl C3H mammary tumor cell cultures was developed to determine whether changes in viral antigen expression and release into culture fluids could be utilized as an *in vitro* measure of modulating drug effect. The 52,000 MW viral envelope glycoprotein (gp52) of the mouse mammary tumor virus (MMTV) was measured in culture fluids of control and drug-treated cultures while cell density was simultaneously determined by cell staining and OD 664 nm determination. While extracellular gp52 levels and cell density progressively increased over 72 hours for control cultures, declines in both parameters provided dual measures of effect for combination [N(phophonacetyl-L-aspartic acid)+ 5-fluorouracil], combination [N(phophonacetyl-L-aspartic acid) + 5-fluoro-5'-deoxyuridine] and single component treatment of this combination. At each treated time point, these combinations begin to produce a greater decline in both cell density and gp52 levels as compared to single drug treatments. These results indicate that N(phophonacetyl-L-aspartic acid) in combination can enhance the effectiveness of single drug.

Keywords ☐ chemosensitivity assay, MMTV, gp52, chemotherapy

The mouse mammary tumor and its associated virus, mouse mammary tumor virus (MMTV), have provided a model system to determine whether plasma levels of the envelope glycoprotein of MMTV (gp52) can be a useful measure of the presence of mammary tumor. Previous results have indicated that gp52 levels of plasma served as a systemic marker for detecting the presence of mammary tumors as well as subsequently monitoring tumor progression or regression (Ritzi *et al*, 1976; Ritzi *et al*, 1977). The ability of plasma gp52 levels to signal tumor regrowths following surgery and to monitor changing tumor status suggested that viral glycoprotein levels might also provide a useful measure of therapeutic drug effect (Ritzi *et al*, 1977; Ritzi *et al*, 1985) We have demonstrated that extracellular levels of gp52 can be utilized as alternative measures of single and combination drug effect (Ritzi and Pyo, 1989; Ritzi and Pyo, 1994).

Recent interest in experimental chemotherapy has centered on the possibility that the cytotoxic anti-tumor effect of known useful drugs may be enhanced and potentiated by metabolic modulating effects of another compound which in some cases may have little or no effect of its own. One such useful modulator appears to be N(phophonacetyl-L-aspartic acid) (PALA), an experimental drug synthesized for the NIH experimental chemotherapy program and only available through this source. PALA has been utilized to enhance the effectiveness of 5-fluorouracil by blocking the conversion of carbamyl phosphate to carbamyl aspartate. This PALA block decreases the endogenous production of uridine monophosphate (UMP); thereby decreasing the UMP pool and theoretically enhancing the incorporation of 5-fluorouracil (5-FU) into RNA, as well as, inhibiting thymidylate synthetase and the DNA synthesis pathway. Results in mouse tumor models have demonstrated that PALA does stimulate incorportion of 5-FU into RNA and PALA treatments have resulted in enhanced anti-tumor effects (Spiegelman *et al*, 1980). For these reasons, PALA was chosen as an in vitro modulator of 5-FU activity to determine whether changing levels of viral antigen would provide a meaningful measure of experimentally modulated therapeutic effect. The data presented herein demonstrated that decrease in extracellular levels of MMTV gp52 could be utilized in this *in vitro* assay as an alternative measure of chemotherapeutic effect to evaluate the molulating nature of drug.

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MATERIALS AND METHODS

Cells

The Mm5mt/cl C3H mouse mammary tumor cell line was obtained from Dr. Ritzi (Texas Tech Health Science Center, USA). Cells in T75 flasks were maintained at 37°C (5.0% CO_2) on Dubelcco modified Eagle minimal essential medium-high glucose (DMEM-HG) containing 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), insulin (250 IU/liter), tylocine (60 g/ml), penicillin (100 U/ml), and streptomycin (100 g/ml). Cells were subcultured at 3 day intervals by trypsinization with a 0.2% trypsin solution containing 5 mM EDTA.

Chemotherapeutic Compounds

5-fluorouracil and 5-fluoro-5'-deoxyuridine were purchased from Sigma Chemical company, St Louis, MO and N(phophonacetyl-L-aspartic acid) (PALA) was obtained from the NIH experimental chemotherapy program and only available through this source. These compounds were dissolved in DMEM-HG without serum.

Simultaneus in vitro measurement of cell density and MMTV gp52 during treatment

The following protocol was utilized to prepare cells for testing and to simultaneously monitor changes in cell growth (cell density) and extracellular gp52 levels following drug treatments for 24, 48, and 72 hours intervals. The Mm5mt/cl C3H mammary tumor cell line was grown in 96 well microplates. For measurements of drug effect, 0.2 ml of cell suspension (2×10^4 cells/ml) was placed in each well. After 24 hours, the medium was removed from each well and a 0.2 ml volume of medium containing test compound was added to each of 6 wells for each concentration tested. Thereafter, cells were washed and stained with methylene blue at 24, 48, and 72 hours after drug addition. Stained cells were read at 664 nm in a Molecular Device microplate reader (Menlo, CA) on days 1, 2 and 3 following the addition of test compound to measure changes in cell density. Untreated control cultures received medium without drug.

The same 96 well cultures were simultaneously used to monitor changes in the expression and release of MMTV gp52. At each time point (0, 24, 48 and 72 hrs), coincident with cell staining, the extracellular culture fluid for each drug concentration and for untreated controls was removed from 6 cell cultures, pooled, and quick frozen at -70°C for gp52 radioimmunoassay (RIA). In addition, medium which was not exposed to cell cultures was quick frozen as a control. Samples were saved and were simultaneously assayed in triplicated by gp52 RIA.

Methylene Blue staining of adherent cells

Adherent cells were fixed with 0.1 ml of 10% formalin in phosphate buffer solution (0.1 M, pH 7.5). The cell monolayers were washed using borate buffer (0.1 M, pH 8.4) and stained for 30 minutes with 0.5% methylene blue. The cells were washed 4 times with borate buffer and then allowed to dry at room temperature. Each well was filled with 200 µl of 0.1 N HCl. This treatment eluted the methylene blue dye from the stained adherent cells and the resulting solution was measure for OD at 664 nm in a Molecular Device microplate reader.

Radioimmunoassay of MMTV gp52

The 52,000 MW envelope glycoprotein, MMTV gp52, purified by concanavalin affinity chromatography from C3H tissue culture-derived virus and iodinated by the chloramine T procedure was used for RIA as described previously (Ritzi et. al 1992). The sensitivity of this gp52 assay was 0.5 to 1.0 ng/ml and was performed in the presence of the protease inhibitor Trasylol (500 kallikrein inhibiton units/ml). Determinations of gp52 levels are presented as the mean and SEM of triplicates for all times and conditions tested.

Statistical Methods

Differences in levels of gp52 antigen or OD 664 nm measurements were evaluated for significance employing a two-tailed Student's t test. Differences were held significant at $p < 0.05$.

RESULTS AND DISCUSSION

Since PALA has been known to stimulate incorporation of 5-FU into RNA and PALA treatments enhanced the effect of anticancer drugs, PALA may influence on changing levels of viral antigen. To test the effect of PALA on the levels of viral antigen, C3H mammary tumor cells were divided into 3 experimental and one control group for in vitro testing. At zero time, replicate control cultures received fresh growth medium, a second group received 5-FU (2×10^{-5} mg/ml), a third group received PALA alone (6×10^{-3} mg/ml), and a fourth group was treated for 2 hours with PALA followed by a combined regimen of PALA plus 5-FU. The concentrations used are the drug concentration required for 50% reductions in viral antigen levels (ED_{50}) (Ritzi and Pyo, 1989). The effect of these treatments was assessed by monitoring cell density and viral antigen release at 24, 48, and 72 hours post-

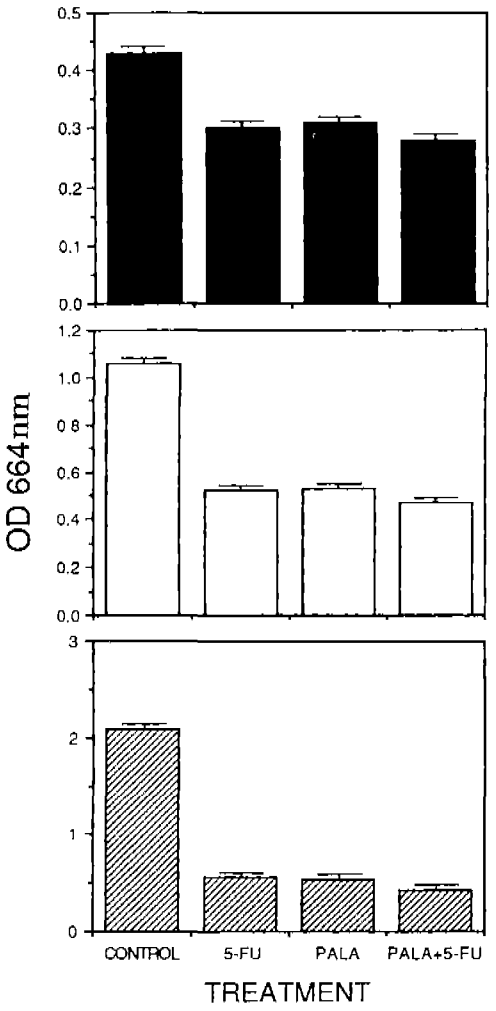

Fig. 1. PALA modulation of the 5-flurouracil effect on cell density. As a measure of cell density, OD 664 nm has been determined for C3H control mammary tumor cells and groups receiving 5-FU, PALA, or PALA primed combination 5-FU plus PALA treatment. Determinations are presented as comparative histograms for 24 hour cultures (top: solid histograms), 48 hour cultures (middle: open histograms) and 72 hour cultures (bottom: cross-hatched histograms). The results are mean±SEM for quintuplicates from a representative experiment.

treatment. The cell density measurements for controls, single treatments and PALA plus 5-FU are presented as histograms in Fig. 1. The results depicted at 24 hours demonstrate a decrease in cell density for each of the three treatments as compared to control. This decrease for each group was statistically significant at $p \leq 0.01$ (paired Student's t test). However, statistical analysis did not support a difference between individual treatments and PALA plus 5-FU treatment at this early 24 hour point in treatment. At 48 hours, a further cytoreductive effect was noted for all these experimental groups as compared to control ($p \leq 0.01$); however, at 48 hours the combination PALA plus 5-FU begins to produce a slightly greater decline in cell density as compared to 5-FU alone. While this difference is small, it is statistically significant at $p \leq 0.05$. The cytoreductive effect at 72 hours is even greater for treatments ($p \leq 0.01$). The combination PALA plus 5-FU continues to be slightly more effective than 5-FU alone and 72 hour statistical analysis more strongly supports this difference ($p \leq 0.01$). These results indicate that both PALA and 5-FU are effective agents which inhibit tumor cell growth and that PALA in combination can enhance the effectiveness of 5-FU. These concentration of PALA (an ED_{50} dose) was high enough to produce an effect of its own. Dosage of the modulator PALA$<5.0 \times 10^{-3}$ mg/ml did not elicit an antitumor effect.

These effects on cell density were further compared with extracellular MMTV gp52 levels. Radioimmunoassay data for each group is presented in Fig. 2. These data suggest a striking difference in the time frame for detecting a change in gp52 level as compared to the previously detected changes in cell density. At 24 hours, all experimental groups demonstrate a decline in gp52 level as compared to control but these declines were small and not statistically significant for PALA or 5-FU; however, comparison of combination PALA plus 5-FU treatment with 5-FU alone revealed an early highly significant decline in gp52 level with PALA plus 5-FU treatment ($p \leq 0.01$). At 48 hours, the decline in gp52 with PALA plus 5-FU treatment was significant as compared to control ($p \leq 0.05$). This was the only treatment which significantly reduced gp52 levels at 48 hours. In the lower portion of Fig. 2, the 72 hour cross-hatched histograms illustrate that all three treatments have significantly lowered gp52 levels as compared to control ($p \leq 0.05$ to $p \leq 0.01$) and that the lowest mean was achieved with PALA plus 5-FU treatment. These results indicate that the PALA plus 5-FU effect was detected earlier by statistically significant changes in MMTV gp52 than by changes in cell density ; however, with 72 hours of treatment cell density also became an indicator of enhanced combination therapeutic effect. We also examined 5-fluoro-5'-deoxyuridine (5'-dFurd), the derivative of 5-FU, to determine whether PALA modulates metabolically the chemotherapeutic effect of 5'dFurd. As shown in Fig. 3, extracellular MMTV gp52 levels were decreased in a similar manner like treatment of PALA plus 5-FU. Concordantly, cell density was decreased in a parallel fashion with time (data not shown). These results suggest that for some drugs

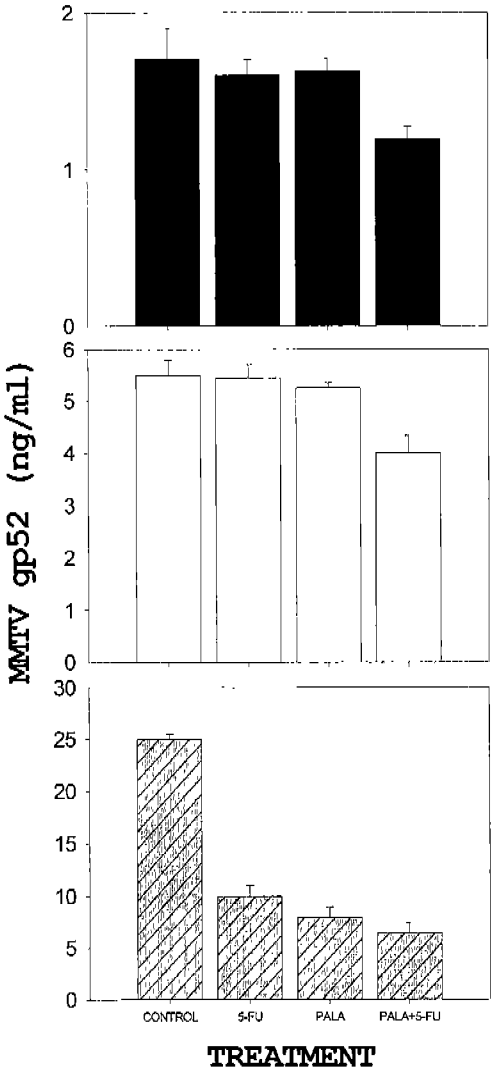

Fig. 2. PALA modulation of the 5-flurouracil effect on extracellular MMTV gp52 levles. Levels of extracellular MMTV gp52 have been determined by radioimmunoassay for the groups described in figure 1. These levels are are presented as comparative histograms for 24 hour cultures (top: solid histograms), 48 hour cultures (middle: open histograms) and 72 hour cultures (bottom: cross-hatched histograms). The results are mean±SEM for triplicates from a representative experiment.

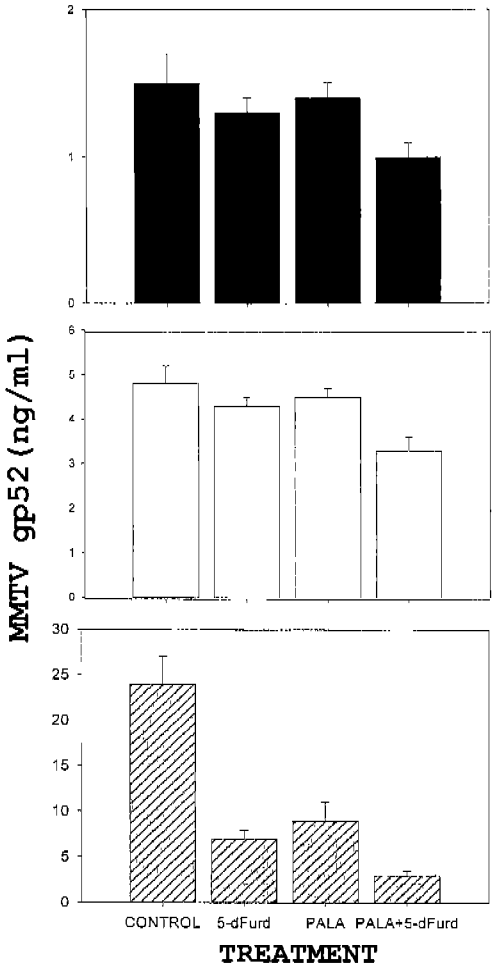

Fig. 3. PALA modulation of the 5-fluro-5'-deoxyuridine effect on extracellular MMTV gp52 levles. Levels of extracellular MMTV gp52 have been determined by radioimmunoassay for the groups described in Fig. 1. These levels are are presented as comparative histograms for 24 hour cultures (top: solid histograms), 48 hour cultures (middle: open histograms) and 72 hour cultures (bottom: cross-hatched histograms). The results are mean±SEM for triplicates from a representative experiment.

and treatments viral gp52 levels may be a more sensitive measure of drug effect than classical measures of cytoreduction. In addition, the cytotoxic anti-tumor effect of 5-FU or 5'dFurd may be enhanced and potentiated by metabolic modulating effects of PALA.

Results with drug treatment (Fig. 1 and Fig. 2) demonstrated coordinate declines in gp52 and cell density which provided dual measurements of in vitro anti-tumor cell effect. However, while gp52 levels have, in general, correlated with changes in cell density, differences in kinetics of effects on gp52 expression suggest that in some instances, changing gp52 levels may provide insight into mechanisms and interactions involved in drug effect which are not afforded strictly by measures of cell death or cell growth inhibition. One such disproportionate effect has been reported. Yagi and coworkers demonstrated that PTT.119, the new experimental antitumor drug [p-fluoro-L- Phe-m-bis-(2-chloroethyl) amino-L-Phe-Met-ethoxy HCl], had a greater disproportionate effect on MMTV replication than on tumor cell killing in the continuously infected MJY-alpha mammary tumor cell line (Yagi *et al*, 1986).

Our *in vitro* assay is proposed as but one tool in the drug screening process. The historical approach has relied heavily upon *in vivo* animal tumor models for screening and present day chemotherapists strongly argue for continuing this approach to anti-tumor drug selection (Lane, 1979). In addition, newly available continuous human mammary tumor cell lines are being used in cytotoxicity assays for drug screening *in vitro* and some investigators argue that these cell lines may eventually take the place of *in vivo* animal testing (Boyd, 1984). Our *in vitro* assay with a uniform continuous murine mammary tumor cell line may be problematic in displaying differences in drug effect as compared to human cells; however, this *in vitro* system offers uniformity, standardization, and a simultaneous dual measure of therapeutic effect not afforded by other in vitro human cell assays. This *in vitro* assay, like all *in vitro* assays, has the disadvantage that compounds may not be metabolized and converted to active therapeutic metabolites as would occur *in vivo* and difficulties exist in extrapolating *in vitro* dosage levels to clinically relevant treatment levels.

As an extension of earlier our study, present data further support that diminished gp52 levels can provide a sensitive measure of in vitro chemotherapeutic effect that may be useful in experimental attempts to maximize presently effective treatment and to evaluate new drugs and new therapeutic protocols. In addition, this use of viral antigen as a measure of therapeutic effect should aid in the design of protocols to improve breast cancer management.

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