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Interactions of Prednisolone and Other Immunosuppressants Used in Dual Treatment of Systemic Lupus Erythematosus in Lymphocyte Proliferation Assays

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Abstract

Systemic lupus erythematosus is an autoimmune disease primarily affecting women. Currently, systemic lupus erythematosus therapy is suboptimal due to adverse effects of immunosuppressants, particularly corticosteroids. This study determines the single effects of prednisolone, dehydroepiandrosterone, bromocriptine, tamoxifen, mycophenolic acid, 2-chloro-2'-deoxyadenosine, azathioprine, and chloroquine on lectin-stimulated proliferation of human T lymphocytes, as well as determining whether there are interactions in the joint effects of prednisolone and these agents. The T lymphocytes from the whole blood of 10 middle-aged women were stimulated by phytohemagglutinin and cultured with varying drug concentrations. The Hill function was used to evaluate single-drug response data. Isobolograms were constructed to qualitatively analyze interactions. Parametric analysis based on competitive and noncompetitive interaction models was further applied to quantify the joint interactions and predict steroid-sparing potential. The surface interaction parameter (ψ) estimated from parametric analysis was in concordance with isobolographic inspection for all interactions studied. All interactions favored the noncompetitive model. Results suggest that dehydroepiandrosterone is additive in its effect with prednisolone, whereas tamoxifen interacts synergistically, both providing steroid-sparing effects. Novel immuno-suppressants such as mycophenolic acid may still provide added pharmacologic benefit during therapy despite a slight antagonistic interaction with prednisolone. These studies help rationalize actual or potential use of other drugs with prednisolone in the treatment of systemic lupus erythematosus.

Keywords

Drug interactions; immunosuppressants; prednisolone; systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic, fluctuating, multisystem autoimmune disease with a diversity of clinical presentations. Disease severity ranges from mild arthritis and cutaneous symptoms to severe inflammatory pain and organ damage. Abnormal immunologic function and formation of antibodies against “self” antigens underlie its

pathogenesis. The reported female to male ratio is 10:1,¹ and peak incidence in women occurs during middle age. Although the etiology of SLE is still unraveled, a possible hormonal role^{2,3} has been implicated based on the predominance of the disease in females and the immunomodulatory effects of certain hormones.

The mainstay of lupus management is immunosuppressive therapy consisting mainly of corticosteroids.^{4,5} Prednisone doses during maintenance therapy range from low (10 mg daily) to moderate (< 100 mg daily). Mini-pulses of prednisone 100 to 200 mg/d for 2 to 5 days are given for rapid control of active disease, and methylprednisolone bolus doses of 1000 mg IV for 4 days are administered for acute lupus nephritis. Other cytotoxic agents such as cyclophosphamide and azathioprine^{6,7} have become standard adjunctive treatment for the more severe manifestations of SLE. In addition, antimalarial agents⁸ such as chloroquine and hydroxychloroquine have shown effectiveness in symptomatic relief and underlying disease activity.

Therapy of SLE is currently suboptimal due to the adverse effects from corticosteroid therapy such as hyperglycemia, hypertension, hyperlipidemia, avascular necrosis, osteoporosis, and muscular atrophy. These side effects have a profound effect on the quality of life of lupus patients.

Agents such as dehydroepiandrosterone (DHEA),^{9,10} tamoxifen,¹¹⁻¹³ bromocriptine,^{14,15} mycophenolic acid (MPA),¹⁶⁻¹⁸ and 2-chloro-2'-deoxyadenosine (2-CDA)¹⁹ have shown positive therapeutic outcomes in human and/or murine SLE models and have relatively safe side effect profiles. Combination therapy of prednisolone with relatively safe disease-modifying compounds that are additive or synergistic in their therapeutic effects may afford adequate immunosuppression while sparing patients high steroid doses and their adverse effects. The immunodynamic interactions of prednisolone with many current standard adjunctive therapies have not been established.

This study determines the single and joint effects of prednisolone, as well as other agents, on lectin-stimulated proliferation of human T lymphocytes ex vivo. Prednisolone is thought to exert its immunosuppressive action on lupus mainly through inhibiting the expression of the cytokine IL-2 causing subsequent antagonism of T lymphocyte proliferation.⁵ The mitogen-induced lymphocyte proliferation assay, is the most widely used method to assess the activity of immunosuppressive compounds. We used the whole-blood lymphocyte proliferation (WBLP) assay, as it mimics the natural environment and requires small volumes of blood.²⁰

There are several approaches to determine the nature of pharmacodynamic interactions between 2 or more compounds. The classic isobologram²¹ is the simplest and is very useful for qualitative data analysis. However, it lacks a summary parameter describing the entire surface of interaction. A parametric method based on an extension of the competitive and noncompetitive approach proposed by Ariens and Simonis^{22,23} is more complex, but in addition to providing a quantitative summary parameter of the interaction surface, ψ , it provides a more mechanistic interpretation of the joint interaction.²⁴ This article uses both

methods to assess the dual interactions between prednisolone and the other compounds using WBLP assays.

METHODS

Subjects

Ten healthy middle-aged drug-free women who were not taking birth control pills, ages 30 to 47 years, were recruited. Blood was drawn at 9 AM on the day of the experiment.

Materials

All chemicals were purchased from Sigma (St Louis, Mo). Stock solutions of prednisolone, DHEA, tamoxifen, bromocriptine, azathioprine, choroquine, MPA, and 2-CDA were prepared in ethanol, methanol, or dimethyl sulfoxide (DMSO) and stored at -20°C . The stock solutions were prepared to ensure the solvent was at optimal concentrations so as to solubilize the greatest mass of drug but to remain inert to T lymphocyte proliferation.

Whole-Blood Lymphocyte Proliferation Assay

This procedure was adapted from Piekoszewski et al.²⁵ Blood samples (20 mL) were diluted 1:20 (v/v) with RPMI 1640 supplement with 10 $\mu\text{g/mL}$ streptomycin, 100 U/mL penicillin, 2 mM L-glutamine, 20 mM HEPES, and 0.25 mM 2-mercaptoethanol (Gibco, NY). Diluted blood was plated in 96-well plates at 165 $\mu\text{L/well}$ with drug (20 μL) at various concentrations. Proliferation was induced by phytohemagglutinin (PHA) (15 $\mu\text{L/well}$) at an optimized concentration of 3 $\mu\text{g/mL/well}$. The drug concentration in each well was calculated based on the final volume of 200 $\mu\text{L/well}$. Cell cultures were incubated at 37°C in a 7.5% CO_2 -humidified air incubator for 72 hours. Cultures were pulsed with 1 μCi of ^3H -thymidine per well and incubated for 20 hours. The cells were then harvested into 96-well microplate filters. The filters were bleached with 3% hydrogen peroxide and dried with pure ethanol. Radioactivity in the filters was counted in a Top CountTM Microplate Scintillation Counter (Packard, Conn) with 25 μL scintillation fluid. The counts per minute (CPM) values were analyzed.

Data Normalization

Background CPM (no drug and no mitogen) and maximum CPM (mitogen alone) were used to normalize CPM of drug response (CPM_{drug}) as follows:

$$\%S_{\text{max}} = 100 \left[\frac{(\text{CPM}_{\text{drug}} - \text{CPM}_{\text{back}})}{(\text{CPM}_{\text{max}} - \text{CPM}_{\text{back}})} \right], \quad (1)$$

where $\%S_{\text{max}}$ is the percentage of drug response with respect to maximal lymphocyte proliferation.

Single-Drug Response Studies

Prednisolone, DHEA, bromocriptine, tamoxifen, MPA, 2-CDA, azathioprine, and chloroquine were studied separately. Background, maximum response, and 10 concentrations spanning 4 log units for each drug were examined in 4 replicates.

Interaction Studies

Five concentrations producing between 25% and 75% $S_{\max}$ in the single-drug response study were selected for each drug in the combination. Concentrations of drug A (prednisolone) were combined with concentrations of drug B (second drug) in 5 ratios—1:1, 1:2, 2:1, 1:4, and 4:1 (v/v)—producing a total of 25 different combinations of concentrations in each interaction study. Four replicates were made for each combination by transferring the same volume of already mixed drug cocktails to 4 wells in a 96-well microplate. Both single and interaction studies were done using the same blood sample collected on the same day to avoid intrasubject variability in measurements.

Single-Drug Response Analysis

Data from single-drug response for all drugs were fitted using

$$\%S_{\max} = 100 \left(1 - \frac{I_{\max} \cdot C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}} \right) \quad (2)$$

where $I_{\max}$ is a capacity term representing the maximum fractional inhibitory effect achievable by the drug whose value ranges from 0 to 1, IC_{50} is the molar drug concentration inhibiting 50% of the $S_{\max}$, C is the molar concentration of drug, and γ is the Hill coefficient. The $I_{\max}$ was set to 1 for all drugs. Adapt II software²⁶ was used to fit values of IC_{50} and γ from single-drug response data.

Interaction Data Analysis

Isobolograms were constructed to qualitatively assess dual interactions at 1 effect level—in this case, 50% inhibition. The classical technique is based on the additivity equation from Loewe as described by Gessner,²¹ which assumes the fractional effect contributed from each drug is additive to explain the full response from combinations. Graphs were constructed by plotting on the x -axis the ratio of prednisolone concentration in a combination producing 50% of the $S_{\max}$ to the IC_{50} of prednisolone. The same procedure was followed for the y -axis for drug B in the combination. Lateral deviation from the diagonal line qualitatively describes the interaction, with additivity seen when points fall along the diagonal, synergy when points fall below the line, and antagonism when points fall above the line.

Parametric analysis was performed to quantify the potency of pharmacodynamic interaction and assess a mechanistic basis. The pharmacodynamic interaction model used was based on an extension of the competitive and noncompetitive approach first proposed by Ariens and Simonis^{22,23} and later applied by Milad et al²⁷ and Chakraborty and Jusko.²⁴ Because $I_{\max}$ was set to 1 for all drugs, the competitive model applied simplifies to

$$\%S_{max}=100 \left[1 - \frac{\left(A / \left(\Psi \bullet IC_{50,A} \right) \right)^{\gamma_A} + \left(B / \left(\Psi \bullet IC_{50,B} \right) \right)^{\gamma_B}}{\left(A / \left(\Psi \bullet IC_{50,A} \right) \right)^{\gamma_A} + \left(B / \left(\Psi \bullet IC_{50,B} \right) \right)^{\gamma_B} + 1} \right], \quad (3)$$

where ψ is a quantitative summary parameter for the entire interaction surface.

The pharmacodynamic model based on noncompetitive interaction is

$$\%S_{max}=100 \left[1 - \frac{\left(\frac{A^{\gamma_A}}{(\Psi \bullet IC_{50,A})^{\gamma_A}} \right) + \left(\frac{B^{\gamma_B}}{(\Psi \bullet IC_{50,B})^{\gamma_B}} \right) + \left(\frac{A^{\gamma_A}}{(\Psi \bullet IC_{50,A})^{\gamma_A}} \right) \bullet \left(\frac{B^{\gamma_B}}{(\Psi \bullet IC_{50,B})^{\gamma_B}} \right)}{\left(\frac{A^{\gamma_A}}{(\Psi \bullet IC_{50,A})^{\gamma_A}} \right) + \left(\frac{B^{\gamma_B}}{(\Psi \bullet IC_{50,B})^{\gamma_B}} \right) + \left(\frac{A^{\gamma_A}}{(\Psi \bullet IC_{50,A})^{\gamma_A}} \right) \bullet \left(\frac{B^{\gamma_B}}{(\Psi \bullet IC_{50,B})^{\gamma_B}} \right) + 1} \right] \quad (4)$$

Individual dynamic parameters of drugs A and B are obtained from equation (2) from the single-drug response data for each drug and then fixed in equations (3) and (4). The Adapt II program was used to perform nonlinear regression fitting of interaction data. Unlike the isobologram technique, which was applied only to 50% effect-level interaction data, equations (3) and (4) were applied to the whole set of interaction data of drugs A and B at various concentrations and ratios. This yields ψ for the whole interaction surface. If ψ equals 1, the combination is additive, whereas ψ greater than 1 indicates synergism, and ψ less than 1 indicates antagonism. Results obtained are expressed as mean $\pm$ SD.

Model selection (competitive vs. noncompetitive) and goodness of fit were determined by Akaike (AIC) and residual sum of squares (RSS) criteria.

A therapeutic molar concentration of prednisolone was used in equation (3) or (4), depending on which model was selected to characterize a dual interaction, with the concentration of drug B set to 0 to determine a therapeutically relevant percent effect of drug A alone. Using the same prednisolone concentration, a concentration of drug B expected to be observed clinically was employed in the same equation jointly. The 2 results were then compared to determine whether drug B is expected to modify the therapeutic activity of prednisolone at clinical exposures.

RESULTS

All WBLP studies showed sigmoidal inhibition with increased drug concentrations. Typical data profiles of all single drugs are shown in Figure 1. The Hill function captured response data well for all immunosuppressants studied, as indicated by visible inspection of goodness of fit, low sum of squared residuals, and low coefficient of variation (data not shown). The R^2 was as high as 0.98 for prednisolone and as low as 0.80 for bromocriptine. As shown in Figure 1, all drugs with the exception of bromocriptine, DHEA, and azathioprine showed complete suppression ($I_{max} = 1$) of T lymphocyte proliferation.

As shown in Table I, drugs studied varied in immunosuppressive potency. Prednisolone (mean IC_{50} of 0.03 μ M) was on the same order of potency as MPA (0.08 μ M) and 2-CDA (0.06 μ M). Azathioprine (1.4 μ M) was about 10 times more potent than tamoxifen (18 μ M),

which showed slightly more inhibitory activity than chloroquine (20 μM). DHEA (56 μM) was about twice as potent as bromocriptine (120 μM), which showed the lowest immunosuppressive activity of all drugs studied. The change of response to increasing drug concentrations, given by the value of the Hill coefficient γ , was gradual for drugs such as bromocriptine ($\gamma = 0.3$), steep for drugs such as mycophenolic acid ($\gamma = 2.8$), and steepest for tamoxifen ($\gamma = 4.7$). Prednisolone ($\gamma = 1.20$) and DHEA ($\gamma = 0.99$) had moderate slope factors.

Variability in the single-drug response data was generally low for all drugs, as indicated by low standard deviations, except for bromocriptine and azathioprine. The IC_{50} estimates of azathioprine for the 3 subjects studied were 0.32, 2.21, and 1.69 μM , whereas those of bromocriptine for 4 subjects studied were 22.8, 18.4, 209, and 225 μM .

Isobolograms drawn at the 50% effect level are shown in Figure 2. These plots qualitatively characterize the nature of the interaction. Combination data points falling close to the diagonal line indicate no interaction or additivity, whereas those falling clearly above or below the line depict antagonism and synergism. Chloroquine, DHEA, and 2-CDA displayed additivity, with interaction data for all subjects clustered along the diagonal. However, in the azathioprine/prednisolone isobolograph, female subject A (shown in square symbols) exhibited all interaction data points clearly above the additivity line (this subject had a significantly lower IC_{50} for azathioprine compared to 2 other subjects), whereas the 2 other subjects showed interaction points on and slightly below the line of additivity. The MPA and bromocriptine isobolograms show combination data clearly above the line, indicating an antagonistic interaction with prednisolone. Tamoxifen showed a synergistic interaction with prednisolone at the 50% effect level, with combination data falling clearly below the line of additivity.

Table I shows summary mean interaction parameter estimates of ψ , the interaction potency parameter, based on the competitive and noncompetitive pharmacodynamic models for all interactions studied. Variability in the interaction data was low, as indicated by the low standard deviations, except for the azathioprine study. As shown in Table I, all subjects in the DHEA, chloroquine, and 2-CDA studies consistently displayed ψ values close to 1 for both competitive and noncompetitive models. Female subjects in the MPA and bromocriptine interaction studies showed ψ values greater than 1 for both models, whereas subjects in the tamoxifen study consistently showed ψ values less than 1. Subject A in the azathioprine study produced a ψ of 1.9 for the noncompetitive model and 1.4 for the competitive model, and subjects B and C produced ψ estimates of 0.65 and 0.71 for the noncompetitive model and 0.55 and 0.57 for the competitive model.

From Table I and Figure 2, it is seen that estimated values of ψ agree with isobolographic results at the 50% effect level for all interactions studied.

The AIC model-fitting criterion factor was calculated from the competitive and noncompetitive model fittings for each interaction. Lower AIC values serve as criteria for the selection of 1 model over another. With the exception of 2-CDA and chloroquine, AIC data for all subjects were lower for the noncompetitive model. One subject in the 2-CDA

and chloroquine interaction studies showed a lower AIC for the competitive model. The sum of squared residuals (data not shown) was generally lower for the noncompetitive model, indicating better goodness of fit for interaction data. Based on these results as well as documented mechanisms of action, the noncompetitive model was selected to perform further data analyses.

It was essential to ascertain whether the mean ψ values based on the noncompetitive model were significantly different from 1 to qualitatively interpret the nature of the dual interactions. Chloroquine, 2-CDA, and DHEA interactions all showed ψ values slightly above 1, but as shown in Table II, these deviations were determined to be insignificant at $P < .05$. The MPA and bromocriptine interaction ψ values were significantly different from 1 at $P < .05$, indicating antagonism. The tamoxifen interaction was also statistically significant, indicating a slight synergism with prednisolone. Based on the noncompetitive model, subject A in the azathioprine interaction study showed a ψ of 1.9 and a confidence interval of (1.7, 2.1) at $P < .05$, indicating antagonism. The other subjects displayed synergy, with ψ values of 0.65 and 0.71 and confidence intervals of (0.60, 0.69) and (0.66, 0.76) at $P < .05$.

Tables III and IV show the expected immunodynamic response ($\%S_{\max}$) of prednisolone and adjunct agents at clinical concentrations obtainable in a 70-kg person during maintenance and acute SLE therapy. Peak molar concentrations of prednisolone were calculated using doses of 10 and 100 mg^{4,5} for maintenance and acute therapy (as shown in Tables III and IV) and a volume of distribution of 1.5 L/kg.²⁸ The same analysis was performed with a lower prednisolone concentration (0.014 μ M), corresponding to a 5-ng/mL trough concentration reported.²⁹ Doses and volumes of distribution used to calculate molar concentrations of adjunct agents were azathioprine (50 mg,⁷ 0.8 L/kg²⁸), bromocriptine (2.5 mg¹⁴, 2 L/kg²⁸), chloroquine (250 mg,⁸ 115 L/kg²⁸), 2-CDA (6.3 mg,¹⁹ 4.5 L/kg³⁰), tamoxifen (10 mg,¹³ 50 L/kg²⁸), MPA (1 g,³¹ 4 L/kg³²), and DHEA (200 mg,¹⁰ 30 L/kg³³).

The $IC_{50,A}$, γ , and the clinical concentration of prednisolone were used in equation (4) for the noncompetitive model, with the concentration of drug B set to 0 to determine the $\%S_{\max}$ of prednisolone alone. The clinical concentration of drug B, $IC_{50,B}$, γ , and the relevant ψ value calculated for the noncompetitive model from Table I were then employed in the same equation jointly to determine the effect of drug B at clinical levels on the expected response of prednisolone. The new molar concentration of [prednisolone] with [drug B] fixed in the interaction to achieve the same $\%S_{\max}$ of [prednisolone] alone was then calculated to determine the steroid-sparing potential.

As shown in Table III, DHEA and tamoxifen at clinical levels potentiate the inhibitory effects of prednisolone on $\%S_{\max}$. They both exhibit steroid-sparing effects, as indicated by a decrease in [prednisolone] in the interaction to restore the same $\%S_{\max}$ of [prednisolone] alone. Calculated percent steroid sparing for the DHEA interaction ranges from 27% at high [prednisolone] to 93% at low [prednisolone], whereas that of tamoxifen is between 19% and 21%. Chloroquine has no effect on the activity of prednisolone and shows no steroid sparing. Bromocriptine antagonizes the pharmacodynamic response of prednisolone at

clinical concentrations, thus increasing the concentration of steroid required to maintain the same %S_{max} by about 36% to 46%.

At a molar concentration of 2.6 μM observed during acute therapy from a 100-mg dose, prednisolone reduces the %S_{max} of T lymphocytes to slightly below 0.5%, as shown in Table IV. At clinical doses, MPA, azathioprine, and 2-CDA further reduce this %S_{max}, adding to the immunosuppressive action of the steroid. Azathioprine calculations shown in Table IV are based on mean single-drug and interaction data of subjects B and C. Subject A also showed positive results (data not shown in Table IV), despite an antagonistic ψ , with a combined %S_{max} of 0.33% and a prednisolone concentration of 2.2 μM needed to restore %S_{max} to that of 100 mg prednisolone. Steroid sparing for this subject ranged from 15% at high prednisolone concentrations (100-mg dose) to 100% seen with low prednisolone concentrations (5 ng/mL).

DISCUSSION

The cell-mediated autoimmunity of SLE is manifested in the up-regulation of T lymphocyte activity. This results in increased humoral autoimmunity, resulting in autoantibody deposition in target organs and subsequent inflammation. The severity of the disease depends on the site of autoantibody deposition, ranging from mild cutaneous symptoms to more severe manifestations such as proliferative lupus nephritis.

Prednisolone has long been the cornerstone of lupus therapy.⁴ It suppresses T lymphocyte proliferation by inhibiting NF-κB, which subsequently down-regulates IL-2 gene expression.²⁰ Despite the efficacy of prednisolone in arresting the progression of SLE, the steroid confers numerous adverse effects. The current study has shown that therapeutic optimization through steroid dose sparing is achievable in principle by combination therapy with disease-modifying agents possessing immunosuppressive activity and relatively safe side effect profiles.

Tamoxifen, a synthetic nonsteroidal estrogen antagonist used in breast cancer treatment, has been extensively studied on murine SLE models based on the rationale that estrogens accelerate the course of human and murine lupus.³⁴ The exact mechanism by which tamoxifen inhibits T cell mitogenesis is unclear but possibly occurs by increasing the threshold stimulus necessary for mitogenesis by an increase in intracellular Ca⁺⁺ influx.³⁵ Tamoxifen has displayed significant suppression of lymphocyte mitogenesis in the rat spleen in a dose-dependent manner.³⁶ According to the current study, sigmoidal inhibition of human T lymphocytes in whole blood is observed with an estimated IC₅₀ of 18 μM. This IC₅₀ is at a level 10⁴ times greater than that expected to be observed clinically from a 10-mg dose. Despite this, tamoxifen has shown steroid-sparing effects at therapeutic levels due to a slightly synergistic interaction with prednisolone. Based on the proposed model, a 20% steroid-sparing potential was observed at both low and high prednisolone exposures. The model predicts that a 10-mg dose of tamoxifen coadministered with 8 mg of prednisolone produces the same immunodynamic effect as a 10-mg dose of prednisolone alone. Tamoxifen has only been tested on murine SLE models, and no clinical data for its efficacy in human SLE have been reported. Aside from its synergistic interaction with prednisolone,

other factors justify the testing of tamoxifen in humans as an adjunct maintenance SLE therapy. Tamoxifen alleviated disease severity by suppression of autoimmune IgG3 antibodies in NZB $\times$ NZW SLE mice.¹¹ Moreover, its safety profile, as well as its favorable effects on bone mineral density,¹³ serum lipid profile, and cardiovascular disease, may offset the adverse effects of long-term corticosteroid therapy in humans.

Prasterone (DHEA), an endogenous adrenal steroid, was recently approved by the Food and Drug Administration (FDA) for the treatment of SLE. Such patients frequently have lower levels of DHEA.³⁷ Van Vollenhoven et al⁹ showed that supplementation with a dose of 200 mg daily improves the SLE Disease Activity Index and provides steroid-sparing. Our study corroborates these findings. A previous study showed sigmoidal inhibition of T lymphocytes by DHEA and a synergistic interaction with prednisolone in the Sprague-Dawley rat.³⁸ The current study also showed a sigmoidal inhibition pattern on human T lymphocytes and that DHEA is additive in its joint effects with prednisolone. Drug concentrations obtainable from a 200-mg clinical dose are about twice the IC₅₀ of T lymphocyte suppression (Tables I and III). This favorable pharmacodynamic profile confers a steroid-sparing potential of 27% at high and 93% at low prednisolone concentrations (as shown in Table III). The proposed model predicts that a combined dose of 200 mg DHEA and 7.3 mg prednisolone provides the same immunosuppressive effect as 10 mg prednisolone. It has been reported that patients receiving 10 to 30 mg prednisone and 200 mg prasterone daily for 9 months showed a significantly greater reduction in prednisone doses to 7.5 mg/d than placebo.¹⁰

Chloroquine is an antimalarial commonly used as an adjunct agent in SLE maintenance therapy.⁸ Inhibition of T lymphocyte mitogenesis was shown in previous studies.³⁹ However, the current study shows that chloroquine inhibits T lymphocyte proliferation in human whole blood in a concentration-dependent manner and determines the sensitivity (IC₅₀) of pharmacologic response. This agent showed no interaction with prednisolone in the present study and displayed an additive effect. Despite this, at clinical levels seen from a 250-mg dose, no change in the expected response of prednisolone is expected. Due to its large volume of distribution (115 L/kg), clinical blood levels are 100-fold below its IC₅₀ for inhibiting T lymphocyte proliferation *ex vivo*. This suggests that the therapeutic benefit seen clinically in SLE with chloroquine must be through a facet other than direct suppression of T lymphocytes. It has been proposed that the antirheumatic properties of antimalarials result from their slow interference with antigen processing in macrophages and other antigen-presenting cells.⁴⁰

Bromocriptine is a dopamine agonist that is known to inhibit prolactin secretion. The rationale for inclusion of this agent in adjunct SLE therapy was based on its immunomodulating properties and the fact that prolactin was found to exacerbate autoimmune disease and increase autoantibody formation through up-regulation of the immune system.⁴¹ Bromocriptine exerts its immunosuppressive activity through 2 distinct mechanisms. One is by inhibition of prolactin-induced T lymphocyte stimulation, and the second is through its own intrinsic immunosuppressive activity on T lymphocytes.⁴² Although such effects of bromocriptine on human T lymphocytes were previously reported,⁴² this study is the first to quantify an IC₅₀ for its immunosuppressive effect in human whole blood. It was initially thought that the extrinsic mechanism of action might

explain the variability seen in IC_{50} estimates of the 4 subjects in this study. Differing baseline prolactin levels in the female subjects may confer variability in the extrinsic pathway of bromocriptine immunosuppression,^{31,43} thereby affecting its total immunodynamic effect on T lymphocytes. However, significant variability in prolactin levels in healthy women has been correlated with seasonal and circadian time factors but not the day of menstrual cycle.⁴⁴ Because whole blood was collected for all 4 women at approximately the same time of day, the source of variability in bromocriptine IC_{50} values is not clear. This compound showed an antagonistic interaction with prednisolone and a potential increase in steroid dosage requirement by about 40% to maintain the same immunodynamic effect of prednisolone alone. A double-blind study showed that mean SLE flares per month decreased significantly with 2.5 mg daily bromocriptine treatment compared to placebo; however, the absolute number of flares in each group was similar.¹⁴ The clinical efficacy of bromocriptine in maintenance SLE therapy is still unclear.

2-Chloro-2-deoxyadenosine, also known as cladribine, is a nucleoside analog used in treating hairy cell leukemia and multiple sclerosis. This drug is a selective lymphocyte-depleting agent that acts noncompetitively with prednisolone. It is converted intracellularly to its cytotoxic metabolite by deoxycytidine kinase, which then causes DNA strand breaks and subsequent cell death by apoptosis.⁴⁵ Previous studies demonstrated its immunosuppressive action in vitro⁴⁶; however, our study further characterizes this immunodynamic effect ex vivo, with an estimated IC_{50} of 0.06 μM . Moreover, we show that this potent agent has no immunodynamic interaction with prednisolone and that it is additive in its joint effects with prednisolone. This additivity and the fact that 2-CDA is very potent at clinical levels (IC_{50} is approximately equal to clinical concentrations based on a 6-mg IV dose) confers a steroid-sparing potential of about 35% at higher and 100% at lower concentrations of prednisolone, as shown in Table IV. Davis et al¹⁹ reported a positive therapeutic outcome with 2-CDA in a pilot study of 12 patients with acute lupus nephritis. However, the effect was not consistent, and the incidence of cell cytopenia and herpes zoster opportunistic infections was significant. Although it is still unclear whether this compound has an acceptable risk/benefit profile, the current study suggests that the additive interaction of 2-CDA with prednisolone may afford dose reduction for both agents during acute therapy, thereby achieving desirable immunosuppression and possibly reducing dose-limiting adverse effects of both agents.

Mycophenolic acid is a potent cytotoxic immunosuppressive widely used in solid organ transplantation. It is a selective inhibitor of inosine 5'-monophosphate dehydrogenase type II, a de novo purine nucleotide synthesis enzyme expressed in T and B lymphocytes that is up-regulated upon cell activation.⁴⁷ The immunosuppressive action of MPA on human T lymphocytes in vitro is not a novel phenomenon⁴⁸; however, we show concentration-dependent inhibition ex vivo, with an estimated IC_{50} of 0.08 μM . Although it was shown that MPA displays a slight antagonism in its joint action with prednisolone, the effect of this interaction is offset by the fact that MPA is a very potent immunosuppressive, with a 1-g clinical dose producing an expected concentration of about 100 times its IC_{50} for inhibiting T lymphocyte mitogenesis (Tables I and IV). Complete steroid sparing is observed where, as shown in Table IV, a dose of 75 mg of MPA produces the same expected immunosuppressive effect as 100 mg of prednisolone. Aside from its demonstrated safety

and tolerability,¹⁷ its immunosuppressive potency observed at therapeutic doses may explain the clinical success this agent has seen in the treatment of lupus glomerulonephritis via suppression of IgG autoantibody formation.⁴⁹

Azathioprine is a purine analog widely used for the management of acute manifestations of SLE in combination with corticosteroids.⁴ It arrests T lymphocytes in the S phase of the cell cycle by incorporation into replicating DNA and blockage of the de novo pathway of purine synthesis.⁶ The present study corroborates previous findings of in vitro inhibition of T lymphocyte mitogenesis⁴⁸ but also determines an estimated sensitivity (IC_{50}) of sigmoidal inhibition, as shown in Table I. Variability seen in both single-response and interaction data in the azathioprine studies may be explained by its well-documented variability in pharmacogenetics.^{50,51} Azathioprine is transformed in whole fresh human blood to 6-mercaptopurine ex vivo.⁵² The latter is inactivated intracellularly by thiopurine methyltransferase (TPMT). There is genetic polymorphism of TPMT enzymatic activity, where approximately 90% of the population has high activity, 10% has intermediate activity, and about 0.03% has TPMT deficiency.⁵¹ This may explain why subjects B and C showed different single-response profiles, with a considerably higher IC_{50} (about 10-fold higher than subject A) and a higher γ (1.1 and 0.8 compared to 0.3 for subject A). Interestingly, the interaction surface parameter (ψ) revealed subject A having an antagonistic ψ of 1.9, whereas subjects B and C showed a synergistic ψ (0.65). Results suggest that for patients having high activity of TPMT, azathioprine acts synergistically, with prednisolone conferring a steroid-sparing potential between 75% at high and 100% at low prednisolone concentrations. For patients having low activity of TPMT, a slight antagonistic interaction is seen with a steroid-sparing potential of about 15% at high and 100% at low prednisolone concentrations. More data would be needed to confirm these limited results.

Another agent commonly used in the treatment of acute lupus nephritis is cyclophosphamide. Its combined immunodynamic effect with prednisolone has not yet been assessed and, given its widespread use in SLE therapy, should be assessed in future studies. Moreover, the combined effects of other potent immunosuppressants with prednisolone that are commonly used in solid organ transplantation, such as cyclosporine and sirolimus, have been evaluated in previous studies using the whole-blood lymphocyte proliferation assay and were shown to interact highly synergistically.²⁰ Such immunosuppressive combinations have shown success in the clinic and have led to optimal immunosuppression while reducing the morbidity and mortality related to toxicity of these agents. Interactions of interleukin-10 and prednisolone studied in this ex vivo system have been found to be additive.²⁴

Certain features of the proposed pharmacodynamic model based on Ariens and Simonis^{22,23} underscore its quantitative power. Besides assessing the degree of interaction using a single summary parameter ψ , it provides a physiological basis for interaction by indicating to what degree the sensitivity factor (IC_{50}) is affected, leading to a more mechanistic interpretation of joint drug effects.²⁴ A ψ greater or less than 1 signifies the degree of decrease or increase in the sensitivity of drug effects when given jointly. However, this change in sensitivity may not be reflected in the dual immunosuppressive effect if observed human drug concentrations are several-fold above the IC_{50} . For example, in the case of the MPA interaction with prednisolone, the observed clinical concentrations of MPA exceed its IC_{50}

by 100-fold, completely offsetting the decrease in sensitivity (by an antagonistic ψ of 1.46) of the effect. Therefore, to assess whether a pharmacodynamic interaction confers clinical significance, it must be analyzed in the context of the entire exposure range. The proposed model facilitates this requirement by factoring in part of the pharmacokinetics of each drug.

Finally, certain study caveats should be addressed. Although bromocriptine, DHEA, and azathioprine did not completely arrest T lymphocyte proliferation, an $I_{\max}$ of 1 was assumed for these agents. This assumes that these agents can completely arrest T lymphocyte proliferation at higher concentrations than those studied. It was difficult to study these drugs at higher concentrations because of their poor solubility in the whole-blood media. Increasing drug concentrations required higher solvent volumes, and to maintain pharmacologic inertness, solubilizing solvents such as ethanol could not exceed a certain concentration in whole-blood media.²⁵

Second, to determine if the prodrug azathioprine has any intrinsic immunosuppressive activity of its own, it would be necessary to exclude the active metabolite 6'-mercaptapurine, which is not feasible. It would have been preferable for us to have studied the responses to both agents.

Third, of some general concern is whether some of the cytotoxic drugs such as azathioprine and cladribine were exerting their effects in the whole-blood lymphocyte proliferation assays by nonspecific cytotoxicity rather than immunosuppression. It has been reported, however, that such cytotoxic effects primarily occur in the bone marrow.⁵³

Furthermore, although arguably the most prominent, T lymphocyte activity is only one of several facets controlling the underlying pathophysiology of SLE. Macrophage activity may be another component⁵ by which drugs such as chloroquine may exert their inhibitory effects.⁴⁰ Therefore, drugs showing no effect on T lymphocyte activity at clinical doses may not necessarily be ineffective in SLE therapy but may provide therapeutic benefit via other disease-modifying facets.

Lastly, because the interaction studies were performed using whole blood of healthy women, the results obtained in this study may be extrapolated not only to SLE therapy but also to other clinical scenarios where such dual immunosuppressive combinations may be used. Obtaining whole blood of SLE female subjects would have been ideal but was practically difficult. Such patients would have to be drug free, and finding volunteers meeting this inclusion criterion in the study time frame was difficult.

Although further human studies are necessary to confirm the accuracy of these findings in vivo, this study has elucidated some prospects of optimizing SLE pharmacotherapy by use of combination therapy. Cladribine has shown considerable steroid-sparing potential, although its safety profile in SLE treatment is still unclear. Mycophenolic acid has demonstrated safety and efficacy in the clinical setting. Its slightly antagonistic interaction with prednisolone does not preclude concomitant use with prednisolone, as the dual immunodynamic effect far exceeds that of prednisolone alone at clinical doses. Tamoxifen and DHEA both show promising steroid-sparing potential and confer favorable therapeutic effects that antagonize the adverse effects of prednisolone.

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Figure 1.
Single-effect profiles of ex vivo suppression of whole-blood T lymphocyte proliferation versus concentrations for the 8 drugs studied. Lines depict fittings using the Hill equation.

Figure 2.
Isobolographs of prednisolone/drug B interactions. Differently-shaped symbols represent interaction data from different subjects.

Table I

Summary of Single-Response and Interaction Parameter Estimates for Inhibition of T Lymphocyte Proliferation

Drug	Subjects	Hill Function		Interaction Potency (ψ)	
		IC ₅₀ (μ M)	γ	Competitive	Noncompetitive
Prednisolone	10	0.03 $\pm$ 0.014	1.20 $\pm$ 0.30	—	—
Azathioprine	3	1.41 $\pm$ 0.98	0.75 $\pm$ 0.42	0.84 $\pm$ 0.48	1.11 $\pm$ 0.74
Bromocriptine	4	119.0 $\pm$ 100.0	0.28 $\pm$ 0.11	1.39 $\pm$ 0.13	1.76 $\pm$ 0.21
Chloroquine	4	19.50 $\pm$ 2.24	2.66 $\pm$ 0.14	0.94 $\pm$ 0.19	1.02 $\pm$ 0.20
2-CDA	4	0.06 $\pm$ 0.03	2.33 $\pm$ 0.37	0.99 $\pm$ 0.23	1.19 $\pm$ 0.34
DHEA	8	56.60 $\pm$ 22.10	0.99 $\pm$ 0.30	1.07 $\pm$ 0.25	1.11 $\pm$ 0.11
MPA	5	0.08 $\pm$ 0.02	2.81 $\pm$ 0.33	1.35 $\pm$ 0.19	1.46 $\pm$ 0.20
Tamoxifen	9	17.60 $\pm$ 7.21	4.72 $\pm$ 1.38	0.71 $\pm$ 0.14	0.79 $\pm$ 0.15

All results are reported as mean $\pm$ standard deviation. 2-CDA, 2-chloro-2'-deoxyadenosine; DHEA, dehydroepiandrosterone; MPA, mycophenolic acid.

Table II

Statistical and Qualitative Analysis of the Mean Interaction Parameter (ψ) Based on the Noncompetitive Model

Drug Interaction	Mean ψ	Standard Deviation	95% Confidence Interval of Mean ψ	ψ Significantly Different from 1 [*]	Qualitative Nature of Interaction
Prednisolone/bromocriptine	1.76	0.21	[1.34, 2.17]	Yes	Antagonistic
Prednisolone/chloroquine	1.02	0.20	[0.63, 1.41]	No	Additive
Prednisolone/2-CDA	1.19	0.34	[0.53, 1.86]	No	Additive
Prednisolone/DHEA	1.11	0.11	[0.90, 1.32]	No	Additive
Prednisolone/MPA	1.46	0.20	[1.06, 1.85]	Yes	Antagonistic
Prednisolone/tamoxifen	0.75	0.10	[0.55, 0.95]	Yes	Synergistic

2-CDA, 2-chloro-2'-deoxyadenosine; DHEA, dehydroepiandrosterone; MPA, mycophenolic acid.

* $P < .05$.

Table III

Calculated Effects of Second Drugs on Prednisolone %S_{max} at Clinical Exposures During Maintenance SLE Therapy

Maintenance SLE Therapy	[Prednisolone] (μM)	% S _{max} [Prednisolone] Alone	[Drug B] (μM)	% S _{max} [Prednisolone] + [Drug B]	[Prednisolone] + [Drug B] to Restore % S _{max} of [Prednisolone] Alone	% [Prednisolone] Sparing
Prednisolone + DHEA	0.26 ^a	6.19	23.1	4.38	0.19 + 23.10	26.92
Prednisolone + tamoxifen	0.26	6.19	0.01	4.70	0.21 + 0.01	19.23
Prednisolone + chloroquine	0.26	6.19	0.10	6.19	0.26 + 0.10	0.00
Prednisolone + bromocriptine	0.26	6.19	0.05	9.17	0.38 + 0.05	-46.15
Prednisolone + DHEA	0.014 ^b	69.12	23.1	48.92	0.001 + 23.10	92.86
Prednisolone + tamoxifen	0.014	69.12	0.01	62.60	0.01 + 0.01	21.43
Prednisolone + chloroquine	0.014	69.12	0.10	69.12	0.014 + 0.097	0.00
Prednisolone + bromocriptine	0.014	69.12	0.05	74.27	0.019 + 0.055	-35.71

DHEA, dehydroepiandrosterone; SLE, systemic lupus erythematosus.

^aCorresponds to a 10-mg prednisolone dose obtainable in a 70-kg person.

^bCorresponds to a prednisolone trough of 5 ng/mL previously reported.³⁰

Table IVCalculated Effects of Second Drugs on Prednisolone %S_{max} at Clinical Exposures During Acute SLE Therapy

Acute SLE Therapy	[Prednisolone] (μM)	% S _{max} [Prednisolone] Alone	[Drug B] (μM)	% S _{max} [Prednisolone] + [Drug B]	[Prednisolone] + [Drug B] to Restore % S _{max} of [Prednisolone] Alone	% [Prednisolone] Sparing
Prednisolone + azathioprine	2.64 ^a	0.41	3.18	0.07	0.6 + 3.18	77.27
Prednisolone + MPA	2.64	0.41	11.10	0.00	0.00 + 0.85	100.00
Prednisolone + 2-CDA	2.64	0.41	0.07	0.24	1.70 + 0.07	35.61
Prednisolone + azathioprine	0.014 ^b	69.12	3.18	17.53	0.00 + 0.58	100.00
Prednisolone + MPA	0.014	69.12	11.10	7.38E-05	0.00 + 0.09	100.00
Prednisolone + 2-CDA	0.014	69.12	0.07	23.70	0.00 + 0.04	100.00

2-CDA, 2-chloro-2'-deoxyadenosine; DHEA, dehydroepiandrosterone; SLE, systemic lupus erythematosus.

^a Corresponds to a 100-mg prednisolone dose obtainable in a 70-kg person.^b Corresponds to a prednisolone trough of 5 ng/mL as previously reported.³⁰