Cyclosporin A, FK506, Rapamycin: The Use of a Quantitative Analytic Tool to Discriminate Immunosuppressive Drug Interactions

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ABSTRACT

Potent immunosuppressive agents display toxic complications at full therapeutic doses: cyclosporin A (CsA) produces pleiotropic mesenchymal effects with prominent vasculopathy, whereas FK506 displays severe neurotoxicity associated with a similar range of mesenchymal and possibly vasculitic reactions. The concept of drug synergy seeks to exploit combinations of agents that promote each other's immunosuppressive effects. This article presents a quantitative method to assess synergy in vitro and in vivo—the median-effect analysis. Application of this method revealed synergistic interactions of CsA with steroids, an additive effect with azathioprine, and antagonistic effects with FK506 and enisoprost. Clearly, a synergistic therapeutic combination can be clinically useful only if it is not associated with a similar potentiation of toxic complications. Clinical practice has documented the synergistic relation of CsA plus steroids and the antagonistic relation of CsA with FK506 or enisoprost. Clinical trials of rapamycin, which displays extremely potent synergistic effects with CsA both in vitro with human and in vivo with animal immune responses, may afford important new insights for clinical immunosuppression.

Key Words: Cyclosporin A, FK506, rapamycin, median-effect analysis

Because individual immunosuppressive agents display pleiotropic arrays of nonimmunologic toxic complications when used at therapeutic concentrations, synergistic drug combinations proffer an attractive strategy. Yet, the identification of the interaction between two drugs as truly "synergistic" is more complex than it may first appear. Although two decades ago, Berenbaum (1) rigorously defined "synergism," insisting upon dose-response analyses for each drug alone as well as in a fixed combination ratio, recent studies have loosely applied the term to describe the interactions of cyclosporin A (CsA) with azathioprine (2) or its analog mizoribine (3), as well as with FK506 (4). A quantitative approach to this enterprise, the median-effect analysis developed by Ting-Chao Chou (5), has proven to afford inclusive indices to dissect CsA interactions with FK506 (6), rapamycin (RAPA; 7), corticosteroids (dexamethasone, Dexa; 8), mouse anti-rat T-cell receptor monoclonal antibody R73 (9), 6-mercaptopurine (6MP; 8) and enisoprost (EP; 8).

THE MEDIAN-EFFECT METHOD

The median-effect principle of Chou (5) is based on the premise that the effect of each drug is related to its dose by the equation:

\[ \frac{fa}{fu} = \left( \frac{D}{Dm} \right)^m \]  

Equation 1

where \( fa \) and \( fu \) are fractions of the system affected and unaffected by the drug at dose \( D \), \( Dm \) is the dose that produces 50% effect (the median effect), and \( m \) is the Hill coefficient that describes the sigmoidicity of the dose-effect curve. Logarithmic transformation of the equation displays the linear relationship:

\[ \log \left( \frac{fa}{fu} \right) = m \left( \log D - \log Dm \right) \]  

Equation 2

Thus, the plot using a log D abscissa versus a log \( (fa/fu) \) ordinate shows the slope \( m \) and the \( x \) intercept \( \log Dm \) at the point that \( \log \left( \frac{fa}{fu} \right) = 0 \) and \( fa/fu = 0.5 \). The first step in the analysis is to represent the "goodness of fit" of the dose-effect data to this log-log median-effect plot by the linear regression coefficient, \( r \). For the analysis to be valid, the \( r \) value must be at least 0.75. The parameters \( m \), \( Dm \), and \( r \) are calculated for each drug alone and in combination mixtures.

For the purposes of analyzing immunosuppressive drugs, \( fa \) is defined as the percent inhibition of the immune performance for in vitro experiments and as the length of prolongation of mean survival of heterotopic cardiac allografts in treated versus non-immunosuppressed animals divided by 100 (the
number of days that denotes indefinite graft survival) for in vivo experiments; $f_u$ is defined as $(1 - f_a)$.

Synergism, additivity, or antagonism of combined drugs is calculated by the combination index (CI) as defined by Chou and Talalay (10). For any effect level $x$, the CI is defined by the equation:

$$CI_x = \frac{D_{1 \text{ combined} \cdot D_{2 \text{ combined}}}}{D_{1 \text{ alone} \cdot D_{2 \text{ alone}}}}$$

Equation 3

where $D_{1 \text{ combined}}$ and $D_{2 \text{ combined}}$ represent the amount of drug 1 or 2 necessary in a mixture to produce effect $x$, and $D_{1 \text{ alone}}$ and $D_{2 \text{ alone}}$ represent the doses of the drugs required to produce the same effect $x$ when used alone. CI values less than 1.0 suggest synergism; above 1.0, antagonism; and equal to 1.0, additivity. A computer software package developed by Chou and Chou (11) calculates the median-effect parameters and performs computer-simulated analyses of CI values over the entire $f_a$ range from 0.5 to 1.

In Vivo Experimental Methods

The mean survival time of BUF (RT-1)" heterotopic cardiac allografts in untreated WFu (RT-1") animals is about 7 days. For the median-effect analysis, the mean duration of survival in each treatment group is expressed as the number of days the survival of the graft is prolonged beyond 7 days. The graft survivals with serial doses of each drug are plotted by the log-log relation (Equation 2); the $x$ intercept yields the dose of that drug alone necessary to prolong mean allograft survival by 50 days ($D_m$). Comparison of individual versus combination drug dose-effect curves yields combination index values (Equation 3). The observed effect with doses of $D_{1 \text{ combined}}$ and $D_{2 \text{ combined}}$ in the experiments is compared with the calculated amount of each drug alone, $D_{1 \text{ alone}}$ and $D_{2 \text{ alone}}$, to produce the same effect.

In Vitro Experimental Methods

The immunosuppressive interactions on normal human peripheral blood lymphocytes (PBL) were tested on various immune performances, namely proliferative responses to phytohemagglutinin, anti-CD3 monoclonal antibodies, or mixed lymphocyte reaction (MLR), interleukin 2 synthesis, and cell-mediated lympholysis. The experimental plan initially tests the proliferative responses to phytohemagglutinin, anti-CD3 monoclonal antibodies, or mixed lymphocyte reaction (MLR), interleukin 2 synthesis, and cell-mediated lympholysis. For each combination ratio, the drugs are serially diluted at least fourfold. Drugs are added to the cultures at their initiation (6–8).

THE INTERACTION BETWEEN CsA AND FK506

In Vivo Results

The administration of FK506 plus CsA to WFu (RT-1") hosts engrafted with BUF (RT-1") allografts yields CI values ranging from 3.1 with 0.05 mg/kg/day of FK506 and 3 mg/kg/day of CsA to 13.3 with 0.07 of mg/kg/day FK506 and 10 mg/kg/day of CsA. All of these data show an antagonistic relation between these two drugs.

In Vitro Results

The inhibition of DNA proliferation was assessed with CsA (0.98 to 1,000 x 10^{-6} M) combined with FK506 (31.25 to 2,000 x 10^{-6} M). The CI values were consistently less than those predicted by an additive effect. For example, the combination of 3.91 x 10^{-6} M FK506 and 125 x 10^{-6} CsA produced 36.9% inhibition of [3H]thymidine incorporation after phytohemagglutinin (PHA) stimulation. However, that amount of FK506 alone causes 34% inhibition and that amount of CsA alone causes 39.1% inhibition. Even the qualitative analysis shown in Figure 1 documents this antagonism. The median-effect equation predicts that an additive relation of the two drugs should produce 73.1% inhibition. Indeed, an examination of this interaction over the full range shows an isolated zone of synergism at inhibition levels less than 40%, but consistent antagonism over the clinically relevant range of 75 to 95% inhibition (Figure 2A). Similarly, under conditions of anti-CD3 activation, all three combination ratios showed less inhibition than predicted by an additive effect, with extreme antagonism by median-effect analysis. Finally, the MLR of various normal volunteers showed inconsistent indices at different ratios of this drug combination.

FK506 and CsA each inhibit IL-2 synthesis in vitro in a dose-dependent fashion over a narrow dose range. FK506 produces 50% inhibition at doses 10-fold less than CsA, namely $D_m$ values of 4.47 x 10^{-10} M versus 4.87 x 10^{-8} M, respectively. The corresponding slope $m$ values of the dose-effect relationships of FK506 or CsA are similar, namely 1.0 or 1.14, respectively. The drug combination at molar ratios of 1:62.5, 1:250, and 1:500 (FK506:CsA) was consistently less effective than that predicted by the single-drug results. Furthermore, median-effect analysis confirmed an antagonistic interaction between the drugs in vitro: all CI values derived from actual experimental points were greater than 1.0.

THE CsA/RAPA COMBINATION

In Vivo Results

Groups of WFu rats were treated with 0.02, 0.08, 0.16, or 0.32 mg/kg/day of RAPA by continuous i.v.
Cyclosporin A, FK506, and Rapamycin

Figure 1. In vitro effects on PHA-induced (³H)thymidine incorporation by normal human PBL of FK506 (A), Dexe (B), 6MP (C), and EP (D) in combination with CsA. For additive effects, combination results (cross-hatched bars) should appear to represent the cumulative percent inhibition displayed by each component (solid bars); synergism shows more than this amount of inhibition and antagonism, less.

Figure 2. Comparison of CI (Equation 3) values between CsA and FK506, azathioprine (AzA), and RAPA over the entire inhibition range, namely fraction affected to 95%. Panel A shows the effects on anti-CD3-triggered human PBL proliferation, and Panel B shows the effects on the survival of heterotopic BUF heart grafts in WFu hosts.

Figure 3. Logarithmic conversion of median effect analysis of cardiac allograft prolongation data obtained upon the administration of CsA or RAPA i.v.

Infusion via an osmotic pump or CsA by oral gavage at 1.5, 2.0, 3.0, 5.0, or 10.0 mg/kg/day for 14 days after BUF heart transplantation. Figure 3 shows that the in vivo immunosuppressive effects of CsA and RAPA obey the median-effect equation. In vitro RAPA induced 27-fold greater immunosuppressive effect, namely, an equipotency ratio of CsA Dm 10.67 versus RAPA Dm 0.40. None of the hosts receiving a 14-day course of 0.02 mg/kg of RAPA i.v. or CsA, either 2.0 mg/kg p.o. or 1 mg/kg/day i.v., survived more than 12 days, whereas those that received the combination survived more than 50 days, namely a CI = 0.38 for 0.02 mg/kg of RAPA i.v. plus 2.0 mg/kg of CsA p.o., demonstrating pharmacologic synergism. In experiments with kidney allografts, graft survival increased from 12 days in untreated to over 110 days
with the combination of 0.02 mg/kg/day of RAPA and 1.0 mg/kg/day of CsA. Detailed analysis over the entire fa range is illustrated in Figure 2B. The in vitro results suggest that this drug combination permits CsA dose reduction by 3.7-fold and RAPA dose reduction by 8.9-fold.

To exclude the possibility that the action of RAPA is no more than an inhibition of the metabolism or excretion of CsA, the pharmacokinetics of the endopeptide were examined in 10 rats (5 control and 5 RAPA treated) during the seventh day of a continuous i.v. infusion of either vehicle or 0.08 mg/kg/day of RAPA. Neither the clearance rate nor the t\textsubscript{1/2} of CsA was altered by RAPA. These data showing no detectable effect of RAPA upon CsA biodisposition exclude the possibility that the highly significant immunosuppressive synergism is due to a simple pharmacokinetic effect of RAPA to interfere with CsA elimination.

**In Vitro Results**

In addition to the synergistic effects of CsA and RAPA to inhibit proliferation, the drug combination attenuates the generation of cytokotic cells in cell-mediated lympholysis reactions. Although CsA displays eightfold more potent inhibition of cytokotic T lymphocyte generation than RAPA, the combination shows a CI of 0.09 at the 95% inhibition level, allowing the CsA dose to be reduced by 87% and the RAPA dose to be reduced by 99%. Furthermore, this effect was at least partially IL-2 resistant because the drug combination blocked cytokotic T lymphocyte generation by limiting dilution analysis in the presence of optimal amounts of IL-2.

One important trigger of lymphocyte activation is lymphokine binding to cell surface receptors. Two cell targets of lymphokines are MH60.BSF-2 for IL-6 and CTLL-2 for IL-2. Lineweaver-Burk double reciprocal plots of the Michaelis-Menton equation show IL-6 displays a \( K_m = 0.399 \) U/mL, a \( K_i = 2.4 \) ng/mL, and a \( V_{max} = 63.25 \times 10^3 \) cpm to trigger MH60.BSF-2 proliferation. Lineweaver-Burk analysis revealed that RAPA caused noncompetitive inhibition of IL-6 triggering. Similarly, IL-2 shows a \( K_m = 1.13 \) U/mL, a \( K_i = 22.3 \) ng/mL, and a \( V_{max} = 380 \times 10^3 \) cpm for CTLL-2, and Lineweaver-Burk double reciprocal plots reveal that RAPA produces competitive inhibition. The \( Dm \) for CsA was 2.607 ng/mL and for RAPA was 90.9 ng/mL with a CI value of 0.83 at the 95% inhibition level.

**THE CsA/DEXA COMBINATION**

The in vitro inhibitory effects of Dexa on \(^{3}H\)thymidine incorporation by PHA-activated, normal PBL exhibit marked interindividual and intraindividual variability. Figure 1B shows a strongly synergistic interaction between the Dexa-CsA combination ratios of 1:0.83, 1:3.33, and 1:6.66. Computerized simulation of CI revealed marked synergism in three normal subjects with CI values at 50% inhibition from 0.04 to 0.75. However, three individuals showed synergism in MLR assays, with CI values of 0.16 to 0.89 at 50% inhibition; a fourth responder showed consistently antagonistic CI values that varied from 2.4 to 7.756 over the 50 to 95% range of inhibition. The significance of these findings for in vitro immunosuppression is unclear because these CsA/steroid dose ratios are only administered during antirejection therapy.

**THE CsA/6MP COMBINATION**

6MP:CsA weight ratios of 1:1, 1:2, and 2:1 g/mL were tested for in vitro inhibition of \(^{3}H\)thymidine incorporation by PHA-activated, normal PBL from three individuals, all of whom showed similar results (Figure 1C). Although the responses to a few dose ratios displayed greater than additive inhibition of proliferation, namely CI values as low as 0.39, almost all of the CI values at 50% inhibition were about 1.0, demonstrating an additive effect (Figure 2A). Similar results were obtained after OKT3 or MLR stimulation.

**THE CsA/EP COMBINATION**

EP alone displayed a sharply sigmoidal in vitro dose-effect curve, reflecting a narrow therapeutic range of the agent as a single drug. Antagonistic interactions were evident for the 50:1 and 25:1 combination ratios of EP:CsA over the entire range of inhibition (Figure 1D). CI values ranged from 1.10 to 1.25. However, the EP:CSA 100:1 mixture displayed a marginally additive effect when the level of inhibition exceeded 75%; the values ranged from 0.91 to 1.7 for three normal subjects. Similar effects of EP were documented upon OKT3 stimulation with a sigmoidal dose-effect response (m > 1). Although erratic or slight synergism was observed in some volunteers using drug combinations that produced greater than 75% inhibition in OKT3 and MLR activation, there tended to be a generalized antagonism.

**TRIPLE COMBINATIONS OF DRUGS WITH STEROIDS**

CsA/FK506/Dexa

Triple-drug experiments to assess the effect of the corticosteroid Dexa on the antagonistic interaction between FK506 and CsA compared the combination indices of the dual drug FK506/Dexa with the triple-drug FK506/CsA/Dexa combination. In all situations, Dexa tended to exert a positive effect, increasing the percent inhibition of proliferation above the
level predicted based solely upon an additive contribution. Computer-stimulated CI plots as a function of percent inhibition showed that the addition of large amounts of Dexa tended to mitigate the antagonism between FK506 and CsA with CI values ranging from 0.016 to 0.184. However, the findings reinforce the thesis of an intrinsic antagonism between FK506 and CsA: the addition of CsA in a triple-drug FK/CsA/Dexa combination actually reduced the profound synergistic effect achieved with the dual-drug FK506/Dexa combination.

CsA/RAPA/Dexa

PBL from 17 volunteers showed marked synergism with this triple-drug combination, particularly for the inhibition of the potent PHA stimulus. The triple-drug combination tended to show greater efficacy than the CsA-RAPA combination and more consistent synergism than RAPA/Dexa. The capacity of mixtures to reduce the amount of individual drugs necessary to achieve a given effect level was assessed by the dose-reduction index. Although Dexa showed a variable capacity to reduce the CsA dose, RAPA alone permitted 10- to 1,000-fold and in combination with Dexa, 100- to 10,000-fold, dose reduction. Furthermore, the RAPA dose could almost be reduced to a similar extent. These values are obviously greater than the dose-reduction index level of 2.0, which implies an additive relation of a dual-drug or 3.0 for a triple-drug combination, the values that would be predicted if the second or third agent had the same effect as the simple addition of more of the reference drug.

DISCUSSION

Synergism between immunosuppressive agents may allow dose reduction with preserved biologic effect. This concept of dose sparing is distinct from the concept of blending suboptimal drug effects into an effective matrix that underlies the CsA-azathioprine combination. However, the experimental analysis presented here reveals an important flaw of the latter method: the degree of inhibition produced by blended suboptimal drug doses displays marked interindividual variation. These findings explain the clinical strategy to overcome jeopardy to the transplant by quadruple blends, which induce a profound state of immunosuppression that averts allorejection in most patients. The alternate synergistic approach uses combinations of drugs in low doses to avert their toxic effects in such ratios that they potentiate each other's immunosuppressive effects.

The major obstacle to such an enterprise is a model to assess drug interactions that provides clinically relevant information. The median-effect analysis is a mathematical model based upon the mass effect equation. This analysis has been applied to dissect the interactions between antineoplastic agents (13,14) and anti-AIDS drugs (15,16). Application to immunosuppressive agents has yielded important insights into drug interactions with CsA; RAPA and steroids are synergistic, azathioprine is additive, and FK506 and EP are antagonistic. Interestingly, these conclusions correlate with existent clinical experience. Corticosteroids enhance the effect of CsA (17), FK506 impairs CsA efficacy (18), and EP has, if anything, a deleterious effect (unpublished observations). However, the data with corticosteroids in vitro suggest a mitigation of antagonism that is not consistent with clinical effects. This inconsistency is most likely due to the relatively excessive amount of in vitro-added steroid rather than the experimental replacement of Dexamethasone for methylprednisolone. Therefore, conclusions based on in vitro and in vivo data subjected to the median-effect analysis may have valid clinical extrapolations. These quantitative parameters of drug interactions may thus serve as foundations for the design of studies to assess new immunosuppressive agents.

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