ELSEVIER

Antiviral Research 24 (1994) 361–364

Antiviral Research

II In response to Prichard and Shipman's letter to the editor regarding the paper: Efficacy of ganciclovir in combination with zidovudine against cytomegalovirus in vitro and in vivo. Freitas, V.R., Fraser-Smith, E.B., Chiu, S., Michelson, S. and Schatzman, R.C. (1993) Antiviral Res. 21, 301–315.

Elizabeth B. Fraser-Smith*, Sophie Chiu, Seth Michelson, Randall C. Schatzman

Syntex Research, Palo Alto, CA, 94304, USA

(Received 31 March 1994)

As the title of our paper indicates, we studied the interactions of ganciclovir (GCV) in combination with zidovudine (AZT) on cytomegalovirus (CMV), not on myelosuppression. In the discussion, we were careful to distinguish between the lack of antagonism seen with combinations of the two drugs against the virus and the adverse hematological effects seen when the two drugs were combined. We only suggest that GCV can be used in combination with AZT, while treating an opportunistic CMV infection of AIDS patients, without compromising the efficacy of GCV against CMV (see page 314 in Freitas et al., 1993). As we noted in our paper, GCV appears to be able to be safely used with AZT, when the concern is combining the two drugs against CMV, as evidenced by the fact that an additive to synergistic activity was seen in vitro and no adverse effect was seen in vivo. However, clinical investigators as always need to consider the effects of the two drugs on myelosuppression and co-administer them under carefully controlled conditions when leukocyte counts fall too low (we have addressed this issue on page 313 of our paper).

Prichard and Shipman also had concerns regarding our statistical analyses which need to be addressed. In our paper, we apply a statistical analysis which includes a stepwise linear regression and an analysis of co-variance for detecting drug combination effects (interactions). This procedure is presented in its entirety in the data analysis section of the Materials and Methods, together with an accompanying flow

*Corresponding author.

SSDI 0166-3542(94)00036-8

chart. As we stated in our paper, the data were analyzed using both our linearized regression model and a nonlinear regression model (as by Greco et al., 1990) over the entire dose-response surface. In both analyses, the results suggest that no antagonism between GCV and AZT exists. In every instance, both the linear and nonlinear regression models yielded the same conclusions.

We used these two model forms because we were concerned during our initial analyses that many other existing methods, such as the isobologram (Loewe, 1953), the combination index method (Chou and Talalay, 1981) and Prichard and Shipman's method (1990) were looking at drug combination therapy on a point-by-point basis or across a range of given dose ratios, without verifying an overall and consistent response across the entire drug combination response surface. We have misgivings about these approaches for two reasons. The first is that the basis of all statistical theory is that if we assume an error can be made by chance alone, then the chance of making that same type of error after multiple analyses, *without first verifying an overall response*, compounds the experiment-wise error rate beyond acceptable limits. The second is more philosophical, in that if one were to observe two points in the response surface that yield significant deviations from additivity, and if they were in opposite directions, then one would be forced to posit an underlying biological mechanism of interaction (on the molecular level!), that could account for the response being "turned on" at point A but "turned off" at point B. By looking for an overall response surface effect, we are, by definition, more conservative than Drs. Prichard and Shipman, but we focus our attention on biological mechanisms that act consistently over whole dose ranges.

To that end, in our model an overall test for consistent deviation from additivity must be completed *before* any point-by-point analysis is performed. If evidence for consistent supra-additivity exists, the point-by-point comparisons can be performed directly, i.e., at an alpha level of 0.05. If a point-by-point analysis is desired, *even in the face of no evidence of supra-additivity*, then a Bonferroni correction for multiple comparisons is carried out using Dunn's procedure and Fisher's LSD strategy (Kirk, 1982). These procedures adjust the alpha level downward, i.e., 10 point-by-point comparisons would adjust the alpha down to 0.005 to be significant; all values above 0.005 would be accounted for by noise.

It can be quite risky to perform as many comparisons as Drs. Prichard and Shipman did without some additional protection. In the Prichard et al. paper (1991), 60 point-by-point comparisons were made to compare the observed experimental data to calculated additive data without first performing an overall test for consistent deviation from additivity and without any adjustment for multiple comparisons. When making as many comparisons in a single experiment as they did, whether the significant differences obtained are due to real differences in the functions being compared or simply due to the very large number of comparisons being made, the chance of finding differences that are statistically significant but due to chance alone is of great concern. For example, if an experimenter conducts 25 comparisons in an experiment and finds one significant difference at the 0.05 level, he should not put too much faith in the result because he should expect to find $(0.05)\ (25) = 1.25$ significant differences *just by chance alone*. Thus, if an experimenter is answering a

large number of questions with one experiment, it is desirable to have a procedure that indicates whether the differences might be the result of chance alone (Milliken and Johnson, 1984).

Ignoring the fine points of our technique for the moment (they are outlined in detail in our article), several key points should be emphasized: (1) The relationship between the responses and doses for each drug is first determined *over the entire dose-response range*. If either is not linear, an appropriate transformation (usually the log) for doses is made to establish linearity. (2) This "linearity" is the only assumption in our model. When both drugs are active, a stepwise linear regression is used for the additive model of drug–drug interaction. A term indicating any subsidiary effects that cannot be explained by linear additivity is included in the model. Only if that term is significant, only if the additive model is rejected in favor of the non-additive model, do we take this to mean there is synergy or antagonism, i.e., this term indicates a consistent supra-additive effect across the majority of the active range of drugs. (3) Our linear regression method and Greco's nonlinear regression model both carry this overall systematology, and it seems to be unique in our survey of the literature. Our methods require full dose response curves to gain adequate insight into the overall combination interactions one may experience over the response surface. Most other techniques do not. Therefore, Prichard and Shipman's data can not be analyzed in our model in order to attempt to reconcile the apparent differences regarding cytotoxicity.

If one of the drugs alone is not active, our method can still be used to test for potentiation of drug effects by performing an analysis of co-variance (ANCOVA) (Dixon and Massey, 1969) to test for heterogeneity of slopes. These analyses are also carried out over the entire dose range, and consistency of effects are again used to turn on/off the Bonferroni correction.

The results of any method depend critically on the definition of "no interaction". To our understanding, Drs. Prichard and Shipman's method uses "Bliss independence" as its underlying assumption. Their theoretical additive surface is calculated based on this assumption about the mechanisms of the individual inhibitors. However, the evaluation of any interaction should be performed independently of mechanistic information (Berenbaum, 1989 and Suhnel, 1990). Note that Bliss independence cannot be defined for agents which do not demonstrate a maximum effect (Greco, 1992). Basically, the definition of this assumption is that by adding simple drug effects one derives the effect for the combination. The major fallacy in this approach is illustrated in Berenbaum's paper (1977). Since Drs. Prichard and Shipman's method does not require a full dose response curve, the method must require extrapolation to predict an overall dose response surface in order to make conclusions for the deviations from additivity. Such extrapolations must impact the ultimate conclusions of the analysis.

References

Berenbaum, M.C. (1977) Synergy, additivism and antagonism in immunosuppression. A critical review. Clin. Exp. Immunol. 28, 1–18.

Berenbaum, N.C. (1989) What is synergy? Pharmacol. Rev. 41, 93–141.

Chou, T.C. and Talalay, P. (1981) Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur. J. Biochem. 115, 207–216.

Dixon, W.J. and Massey, F.J. Jr. (1969) Introduction to statistical analysis, 3rd ed., McGraw Hill, San Francisco, California.

Freitas, V.R., Fraser-Smith, E.B., Chiu, S., Michelson, S. and Schatzman, R.C. (1993) Efficacy of ganciclovir in combination with zidovudine against cytomegalovirus in vitro and in vivo. Antiviral Res. 21, 301–315.

Greco, W.R., Park, H.S. and Rustum, Y.M. (1990) Application of a new approach for the quantitation of drug synergism to the combination of *cis*-diamminedichloroplatinum and 1-β-D-arabinoturanosyl-cytosine. Cancer Res. 50, 5318–5327.

Greco, W.R., Unkelbach H.D., Poch G, Suhnel J., Kundi, M. and Bodeker, W. (1992) Consensus on concepts and terminology for combined-action assessment: the saariselka agreement. Arch. Complex Environ. Studies 4, 65–69.

Kirk, R.E. (1982) Experimental design: procedures for the behavioral sciences, 2nd ed. Brooks/Cole, Belmont, California.

Loewe, S (1953) The problem of synergism and antagonism of combined drugs. Arzneim-Forsch. 3, 285–290.

Milliken, G.A. and Johnson, D.E. (1984) Analysis of Messy Data. Volume 1: Designed experiments. Van Nostrand Reinhold, New York, New York.

Prichard, M.N. and Shipman, C. Jr. (1990) A three-dimensional model to analyze drug-drug interactions. Antiviral Res. 14, 181–206.

Prichard, M.N., Prichard, L.E., Baguley, W.A., Nassiri, M.R. and Shipman, C. Jr. (1991) Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and zidovudine. Antimicrob. Agents Chemother. 35, 1060–1065.

Suhnel, J. (1990) Evaluation of synergism and antagonism for the combined action of antiviral agents. Antiviral Res. 13, 23–40.