Antiviral Research 145 (2017) 1–5

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Comparison of three dimensional synergistic analyses of percentage versus logarithmic data in antiviral studies

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ARTICLE INFO

Article history:
Received 23 February 2017
Received in revised form
28 June 2017
Accepted 30 June 2017
Available online 1 July 2017

Keywords:
Drug combination
Synergy
Vaccinia
Cidofovir
6-Azauridine

ABSTRACT

Cell culture antiviral experiments were conducted in order to understand the relationship between percentage data generated by plaque reduction (PR) and logarithmic data derived by virus yield reduction (VYR) assays, using three-dimensional MacSynergy II software. The relationship between percentage and logarithmic data has not been investigated previously. Interpretation of drug-drug interactions is based on a Volume of Synergy (VS) calculation, which can be positive (synergy), negative (antagonistic), or neutral (no or minimal interaction). Interactions of two known inhibitors of vaccinia virus replication, cidofovir and 6-azauridine, used in combination by PR assay yielded a VS value of 265, indicative of strong synergy. By VYR, the VS value was only 37, or weak synergy using the same criterion, even though profound $\log_{10}$ reductions in virus titer occurred at multiple drug combinations. These results confirm that the differences in VS values is dependent of the measurement scale, and not that the degree of synergy differed between the assays. We propose that for logarithmic data, the calculated VS values will be lower for significant synergy and antagonism and that volumes of >10 $\mu M^2 \log_{10}$ PFU/ml (or other units such as $\mu M^2 \log_{10}$ genomic equivalents/ml or $\mu M^2 \log_{10}$ copies/ml) and <-10 $\mu M^2 \log_{10}$ PFU/ml are likely to be indicative of strong synergy and strong antagonism, respectively. Data presented here show that the interaction of cidofovir and 6-azauridine was strongly synergistic in vitro.

Various methods have been devised to study and interpret drug-drug interactions. Prior to the advent of computer programs, two-dimensional (2-D) methods were used to approximate the actual three-dimensional (3-D) nature of drug interactions. 2-D methods had their place historically, but 3-D methods have largely replaced them and allow for rigorous analysis of drug-drug interactions over an entire dose-response surface (Prichard and Shipman, 1990). Understanding the shape of the entire 3-D surface is essential to understanding complex drug interactions.

One of the computer software tools developed to evaluate and quantitatively interpret 3-D dose-response surfaces is MacSynergy™ II. This program graphically plots 3-D interactions that fall above or below a neutral surface (baseline). In addition, the program generates an interpretable value referred to as the Volume of Synergy at 95% confidence limits for each set of data or multiple sets of data that are averaged together. For example, this method has been used for interpreting drug-drug interactions for influenza virus infection studies (Ilyushina et al., 2007, 2008; Smee et al., 2009, 2010a, 2010b) using percent mortality data.

A question that has not been addressed since the development of MacSynergy II is how to interpret logarithmic data in comparison to percentage data that is plotted on a linear scale. Percentage data are produced in many assays, such as percentage of viral cytopathology (compared to uninfected cells) or of viral plaques in plaque reduction (PR) assays, or percentage of surviving animals in a group of infected animals. Viral titer data, such as data derived from virus yield reduction (VYR) assays (Tarbet et al., 2014), or of the amount of virus produced in infected animal tissues (Smee et al., 2016), are more appropriately presented on a logarithmic scale. Viral loads determined by qPCR assays are also most appropriately analyzed in logarithmic form (James et al., 2011; Prichard et al., 2011). In the past where analysis of VYR data by MacSynergy II has been performed, the investigators have not generally interpreted the results much beyond declaring interactions as synergistic, antagonistic, or neutral (Tarbet et al., 2012). In contrast, further interpretations of the degree of synergy (or antagonism) have been given for percentage data, such as weak, moderate or strong synergy (or

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http://dx.doi.org/10.1016/j.antiviral.2017.06.022
0166-3542/

antagonism) (Prichard et al., 1992).

The purpose of the present investigation was to better interpret logarithmic data by MacSynergy II by understanding how the results compared to percentage data. In order to do this, we wanted to use the same virus and cell culture but in two different ways, that would produce both percentage and logarithmic data. Vaccinia virus seemed to be a logical choice of virus, since it is a lytic virus that produces cytopathology and distinct plaques in vitro. Virus yields from the infected cells can readily be quantified by plaque assay. For the present investigation we used the PR and VYR assays as means of deriving percentage and logarithmic data, respectively. This required that we also identify two compounds that would inhibit the virus synergistically when used together in cell culture.

A number of compounds have been discovered that exhibit antiviral activity against vaccinia virus in vitro. Three in particular, cidofovir (De Clercq et al., 1987; Smee et al., 2015), tecovirimat (Jordan et al., 2010; Yang et al., 2005), and brincidofovir (Florescu and Keck, 2014; Quenelle et al., 2007) (an orally active prodrug form of cidofovir), have been considered for human treatment of smallpox and monkeypox virus infections, and two of the compounds have been used to treat complications due to smallpox vaccinations (which employs a live vaccinia virus vaccine) (Lederman et al., 2012). Based on commercial availability, we chose cidofovir as one of the drugs to use in combination to treat vaccinia virus infections in vitro. However, the choice of the compound to combine with cidofovir was not obvious. We first investigated ribavirin, an inhibitor of vaccinia virus (Bougie and Bisaillon, 2004; Smee et al., 2001), but found that the two compounds were just weakly synergistic in combination (D.F. Smee, unpublished). Understanding that cidofovir diphosphate (the antiviral active form of cidofovir that inhibits the viral DNA polymerase (Magee et al., 2008) is a competitive inhibitor of deoxycytidine triphosphate (dCTP) in cells, it was hypothesized that a compound that reduces pyrimidine nucleotide pools may synergize with cidofovir. One such compound, 6-azauridine, an inhibitor of de novo pyrimidine biosynthesis (Handschumacher, 1960; Rada and Dragun, 1977) and of vaccinia virus replication (Rada and Blaskovic, 1966), was evaluated, and we found it to be synergistic when combined with cidofovir. Thus, these two compounds were chosen for the present investigation.

The WR strain of vaccinia virus that was used was obtained from the American Type Culture Collection (ATCC, Manassas, VA). It was propagated in MA-104 cells and titrated by plaque assay in Vero 76 cells. Both cell lines (from ATCC) were derived from African green monkey kidney. Cell culture medium to grow the cells was MEM with 5% fetal bovine serum (FBS).

Plaque reduction (PR) assays were performed in 12-well Corning microplates containing confluent 18 h monolayers of Vero 76 cells. This is possible because plaque sizes at three days are small ($\leq$1 mm diameter). Approximately 80 plaque-forming units (PFU) of vaccinia virus were added to aspirated wells for 1 h, with rocking every 5–10 min to increase the extent of virus adsorption. Virus medium was aspirated from the plates followed by addition of compounds at various concentrations in MEM, 2% FBS and 50 μg/mL gentamicin. Three microwells were used for each concentration (or drug combination) or untreated (virus control) cultures. After 72 h the plates were aspirated dry and fixed with 0.2% crystal violet in 5% buffered formalin for 15 min. The dye solution was removed by pipetting, and the plates were rinsed with water. After air drying, the plaques in each well were counted manually with the aid of a magnifying Plaque Viewer (Bellco, Vineland, NJ). Plaque counts were converted to percentages of the average untreated control wells.

A modification of the above procedure was used for the VYR assay. Approximately 240 PFU of vaccinia virus was rocked onto Vero 76 cells followed 1 h later by drug dilutions. This amount of virus caused nearly 100% cytopathic effect in the wells at 72 h. The plates containing infectious medium were frozen at −80 °C for later titration of virus in each well. Later, partly thawed medium in each well (1 mL) was swirled with a micropipet tip to detach and break up the cells. The fluid was collected from each well, using 3 wells per concentration of inhibitor or combination. The samples were each sonicated 1 min, then the samples were individually titrated by plaque assay on fresh monolayers of Vero 76 cells. Virus titers were recorded as $\log_{10}$ PFU/mL.

The data obtained from the assays were plotted in tabular and graphic form. Tabular data were analyzed for synergy by a two-dimensional drug combination index method (Schinazi et al., 1982). By this method, values obtained for drugs in combinations that are lower than mathematically-determined expected values are deemed synergistic. However, with this method there is no interpretation of the degree of synergy obtained. Graphical interpretations of drug-drug interactions for the same data sets as indicated above were made by a three-dimensional method (Prichard and Shipman, 1990) using MacSynergy II software (Prichard et al., 1992). For percentage data, the virus control (VC) and drug combination data from infected cultures were plotted as 100 minus X (where X is the percent plaque count relative to VC). The uninfected cell control (CC) was indicated as 100 minus 0 (since no plaques were present). For logarithmic data, the raw $\log_{10}$ values were plotted, with VC indicated as the average virus titer obtained from that set of data and CC being 0. This enables the program to plot each graph in the correct orientation, and to calculate synergy and antagonism correctly.

General guidelines were established for interpreting the degree of synergy and antagonism for Volume of Synergy values generated by MacSynergy, as follows: 0 to 25, 25 to 50, 50 to 100, and >100 μm^2 unit % calculated values in either a positive or negative direction using MacSynergy software are defined as insignificant synergy or antagonism, minor synergy or antagonism, moderate synergy or antagonism, or strong synergy or antagonism, respectively (Prichard et al., 1992). The interpretation of drug-drug interactions by this method has been based on percentage data (Prichard et al., 1992). Up until now, there have been no general guidelines to help investigators interpret the Volume of Synergy given for logarithmic data.

The results obtained from the PR assays are shown in Table 1. At 64 μM of 6-azauridine, no plaques were present in the wells, regardless of the cidofovir concentration, and very few plaques formed in the presence of 32 μM 6-azauridine. Lower combinations of 6-azauridine combined with various concentrations of cidofovir produced synergistic suppression of viral plaques in the cidofovir concentration range of 16–128 μM. Since this analytical method provides no interpretation of the degree of synergy observed (Schinazi et al., 1982), the same data were plotted and analyzed by MacSynergy II software and analyzed three-dimensionally (Fig. 1). Nearly the same region of synergy was evident as was shown in the shaded area of Table 1. The volume of synergy observed by this interaction was 275, which is interpreted as strong synergy.

6-Azauridine and cidofovir were evaluated for toxicity in uninfected cell monolayers in 96-well microplates, using the same concentrations (and more) alone and in combination that were used in Fig. 1. A neutral red uptake assay was used to quantify cytotoxic effects of the compounds (Smee et al., 2017). 6-Azauridine alone was inhibitory by 35% at 64 μM, whereas uptake inhibition by cidofovir alone at 128 μM was no greater than 5% (Supplemental Fig. S1). The combinations of 6-azauridine and cidofovir did not reduce neutral red uptake beyond what occurred with 6-azauridine alone.

During the same time that plaque reduction assays were being conducted, 12-well plates were infected and samples later processed for evaluation of virus yields. Table 2 shows inhibition of virus yields by the compounds used alone and in combination. By