Synergy evaluation of anti-Herpes Simplex Virus type 1 and 2 compounds acting on different steps of virus life cycle

Elena Criscuolo[a,1], Nicola Clementi[a,*,1], Nicasio Mancini[a,b], Roberto Burioni[a], Marco Miduri[a], Matteo Castelli[a], Massimo Clementi[a,b]

[a] Microbiology and Virology Unit, "Vita-Salute San Raffaele" University, Milan, Italy
[b] Laboratory of Microbiology and Virology, San Raffaele Hospital, Milan, Italy

ABSTRACT

Despite the clinical need of novel and safe anti-herpetic compounds effective for treating both primary infections and reactivations of Herpes Simplex Virus type 1 (HSV-1) and type 2 (HSV-2), the development of novel antivirals approved for clinical administration has been limited in the last decades to improvements of nucleoside analogues compounds. In this context, targeting different steps of the herpesvirus life cycle, including entry and cell-to-cell infection, can represent an important starting point for obtaining more efficient infection inhibition, and for overcoming both drug resistance and toxicity. Under these perspectives, testing possible synergy between drugs currently in clinical use and novel immunotherapeutics, such as neutralizing human monoclonal antibodies, represents a fascinating option. In the study here described we tested for the first-time possible combinations of inhibitors of Herpesvirus DNA synthesis and a human neutralizing IgG able to block also cell-to-cell infection, by analysing experimental results with different mathematical models. The present study clearly highlights the synergism between all anti-herpetic drugs tested in combination with the mAb; this strongly suggests possible reduction of anti-herpetic drugs combined with the IgG for overcoming drug-related side effects, as indicated by Drug Reduction Index.

1. Introduction

The most effective drugs used to treat primary HSV-1 and -2 infections and reactivations are aciclovir (ACV) and its derivatives, and second line drugs such as foscarnet (FOS). It is well known that both first and second line drugs are burdened by drug resistance, especially in immunocompromised subjects (spanning from 0.7% up to 7% in HIV-positive patients and 11% in post-transplant patients) (Piret et al., 2017). However, even in the case of virological response to therapy, drug toxicity can hamper its proper administration, as in the case of their systemic use in newborns suffering for kidney drug toxicity (James and Kimberlin, 2015). Among the molecules exerting antiviral activity so far described, entry inhibitors (inhibiting both virus entry and cell-to-cell virus passage) represent possible novel candidates and monoclonal antibodies (mAbs) of human origin inhibiting entry-related mechanisms are certainly an intriguing option due to their safety profile (Casadevall et al., 2004; Clementi et al., 2017a, 2013; Lipman et al., 2005). It is also known that different HSV viruses show different susceptibility profiles to the standard drugs, therefore the effective inhibitory concentration of a single drug can vary between virus isolates (Clementi et al., 2017b; Leary et al., 2002). It would be reasonable to expect that the effective dose of a single compound currently used for treating herpetic infections can be modulated, and improved, by co-administering other compounds directed against different virus molecular targets. To evaluate the possible synergistic activity of anti-HSV compounds is crucial to test their capability to inhibit virus replication after virus infection. Therefore, the only neutralization test routinely performed for evaluating mAb potency is not enough for fulfilling the whole functional characterisation of the molecule. The candidate we included in this analysis, named IgG#33, is an anti-HSV human neutralizing mAb able to interfere with virus replication after infection both *in vitro* and *in vivo*, as previously described (Clementi et al., 2017b). Also, the susceptibility of virus isolates to antiherpetic drugs (ACV, FOS, ganciclovir GCV and penciclovir PCV) has been evaluated. Then the combination of these drugs has been analysed for the first time by using post-virus entry assay (PEA). For the evaluation of synergistic activity, the analysis has been based on the use of different algorithms, to overcome intrinsic biases and limitations of single models (Greco

et al., 1995a; Odds, 2003). The data obtained demonstrate the synergistic activity of entry inhibitors combined with DNA synthesis inhibitors for blocking HSV replication even after virus entry into target cells.

2. Materials and methods

2.1. Cells and viruses

Vero E6 (Vero C1008, clone E6 - ATCC® CRL-1586TM) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) containing 10% (v/v) foetal bovine serum (FBS). The laboratory strain HSV-2 MS (ATCC® VR-540TM) was used. The HSV-1 LV strain has been previously described (Tognon et al., 1985).

2.2. Antiviral compounds

Human mAb IgG#33 has been previously described (Clementi et al., 2017b). Briefly, it was selected using phage-display (Criscuolo et al., 2017; Henry and Debarbieux, 2012; Solforosi et al., 2012) from peripheral B cells of a donor showing strong serum IgG ELISA reactivity against both HSV-1 and -2 isolates. mAb#33 was selected against HSV-1 and -2 infected Vero E6 cells after deselection against uninfected cells. Three molecular formats of the antibody were tested *in vitro*. IgG1 format showed the best capability to neutralize HSV infection and block cell-to-cell virus transmission. When tested *in vivo*, IgG#33 fully protected mice from both HSV-1 and -2 lethal challenge.

mAb anti-HCV/E2 IgGe137 (Perotti et al., 2008) was also used as HSV-unrelated isotype antibody control in all experiments.

Aciclovir (ACV; 9-[(2-hydroxyethoxymethyl) guanine]), penciclovir (PCV; 2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-6,9-dihydro-3H-purin-6-one) and ganciclovir (GCV; 2-Amino-1,9-dihydro-9-[[2-hydroxy-1-(hydroxymethyl) ethoxy]methyl]-6H-purin-6-one) were all purchased from Sigma-Aldrich Chemical Company and dissolved in DMSO at a concentration of 10 mg/mL. All compounds were stored as single-use aliquots at $-20\,°C$. Foscarnet (FOS; FOSCAVIR® Astrazeneca SpA) was dissolved in water at a concentration of 24 mg/mL. Dilutions were made in DMEM immediately before use.

2.3. Post-entry assay (PEA)

The post-entry assay was adapted from our previous study (Clementi et al., 2017b). Confluent monolayers of Vero E6 were infected with 100 PFU of virus on 24-well TC-treated plates. After 20 min of adsorption at 37 °C, the virus was removed. Cells were then incubated for 46 h in DMEM containing 2% FBS and 0.5% Agarose in the presence of different concentrations of IgG#33 or antiherpetic drugs (ACV, PCV, GCV or FOS), alone or in combination. Cells were fixed and stained with crystal violet dye. Images were acquired at 5-fold magnification. Viral plaques were counted *in silico* using ImageJ 1.50c4 software (Rasband, ImageJ, U.S.N.I.H., Bethesda USA, http://imagej.nih.gov/ij/) and compared to positive infection control to calculate the percent infection inhibition.

2.4. Data analysis

Drug synergism studies were carried out using CompuSyn software version 1.0 (Chou C. N. and Martin N., Paramus, NJ, 2005) and Combenefit software (Di Veroli G. Y. and Fornari C., Cambridge, UK, 2016 (Di Veroli et al., 2016)). CompuSyn program was used to compute a combination index (CI) for drug combinations studied with growth assays and colony formation assays. The Chou-Talalay combination-index method for drug combination is based on the median-effect equation, derived from the mass-action law principle, which is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. This general equation encompasses the Michaelis-Menten, Hill, Henderson-Hasselbalch and Scatchard equations in biochemistry and biophysics. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1) and antagonism (CI > 1) in drug combinations. This theory also provides algorithms for computer simulation of synergism and/or antagonism at any effect and concentration/dose level, as shown by isobolograms and CI values (Chou, 2010, 2006b).

The approach developed in Combenefit software compares *in vitro* experimental data to mathematical models of dose–responses for non-synergistic combinations. In detail, the three classical models, the Loewe (Loewe, 1953, 1926), the Bliss (Bliss, 1939; Webb, 1963) and the Highest Single Agent (HSA) (Mathews Griner et al., 2014; Tan et al., 2012) models have been incorporated. These models have been used extensively in the literature (Greco et al., 1995a; Odds, 2003). Additionally, a new general model, named Synergy, Antagonism or Neutrality Estimation (SANE) model has been developed by the authors to replace them. The methodology used in Combenefit can be summarized as follows. The experimental dose–response surface that delineates combination effects in concentration space, is first read by the software as a matrix of % of the control value across concentrations. Single agent effects are extracted from this data and fitted with a dose response curve. Based on the two single-agent dose response curves, a model-based combination dose–response surface is derived. This surface provides a 'reference' dose–response surface for a non-synergistic (additive/independent) combination, whose characteristics are determined by the selected model (Loewe, Bliss, HAS, SANE). The experimental combination dose response surface is then compared to the model-generated one, resulting in a synergy distribution in concentration space.

Vero E6 cells infected with HSV-1 or HSV-2 were treated with different concentrations of IgG#33, ACV, FOS, PCV and GCV, either alone or in combination. Dose effect curve, Combination Index and DRI plots were generated through CompuSyn. Sinergy distribution in concentration space of different HSV-inhibitors were obtained using Combenefit.

2.5. Statistical analysis

All experiments were carried out in triplicate and the results expressed as mean ± standard deviation and analysed using GraphPad PRISM® (GraphPad Software, San Diego California USA, www.graphpad.com). Combination index (CI) was measured based on mass action law of degree of drug interaction according to Chou and Talalay. It was calculated using formula $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where $(Dx)_1$ and $(Dx)_2$ represents the dose of drug 1 and drug 2 in a combination which were required to achieve the same efficacy as that of drug 1 (D1) and drug 2 (D2) when used alone (Chou, 2006a). Drug-reduction index (DRI) was also obtained with CompuSyn software using the formula $(DRI)_n = (Dx)_n/(D)_n$ where $(Dx)_n$ alone inhibit x% and n drugs in combination, $D_n = [(D)_n]$ inhibit x%.

Combenefit instead provides a set of metrics (or scores) which captures information about the synergy distribution to facilitate comparison between the four models used. These include metrics such as the maximum synergy, the integrated and the weighted integrated synergy and concentration value at which synergy is most dense.

3. Results

3.1. Phenotypic assay for the evaluation of antiherpetic drugs susceptibility

PEA was performed according to Leary et al. (2002) to evaluate the susceptibility of HSV-1 LV and HSV-2 MS to antiherpetic drugs in Vero E6 cells after virus entry. IC_{50} values obtained for HSV-1 were lower than HSV-2 for ACV and PCV, but not GCV and FOS (Table 1, Fig. S1). On the other hand, IgG#33 biological activity against HSV tested strains has been already described using both neutralization assays,