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COMBINED ANTIVIRAL EFFECTS OF FLAVONOIDS AND 5-ETHYL-2′-DEOXYURIDINE ON THE MULTIPLICATION OF HERPESVIRUSES

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Summary. — The combined antiviral effects of quercetin, quercitrin and 5-ethyl-2'-deoxyuridine on the multiplication of herpes simplex virus type 1 (HSV-1) and pseudorabies virus were studied in vitro by the yield reduction test. Quercetin in combination with 5-ethyl--2′-deoxyuridine had an additive antiviral effect on either herpesvirus. The combined application of quercitrin and 5-ethyl-2′--deoxyuridine showed a synergic effect on pseudorabies virus.

Key words: quercetin; quercitrin; 5-ethyl-2′-deoxyuridine; combined antiviral effect; herpes simplex virus type 1; pseudorabies virus

Introduction

We demonstrated earlier that certain naturally occurring flavonoids have antiviral activity in cell cultures (Bakay *et al.*, 1968, Béládi *et al.*, 1977). The antiviral effect of flavonoids in experimental animals had been described previously (Cutting *et al.*, 1949, 1953); we found later that these compounds may exert a protective effect against Mengo virus in mice (Veckenstedt *et al.*, 1978). The flavonoids we tested were effective mainly against herpesviruses. It is known that 5-substituted 2′-deoxyuridine derivatives are good inhibitors of the multiplication of herpes simplex virus, and some of these drugs have already found use in local therapy in man (De Clerq, 1979). Previously we studied the antiviral effects of some 5-substituted-2′-deoxyuridine analogues both *in vitro* and *in vivo* (Mucsi *et al.*, 1979). It is known that drug resistant mutants of HSV exist and their resistance develops on the basis of different mechanisms (Field *et al.*, 1981; Larder *et al.*, 1983). Combined application of antiviral compounds may help to prevent the development of resistant mutants and the combinations of drugs should be more effective if the mode of action of the compounds is different (Wigand and Hassinger, 1980; Allen *et al.*, 1982). Since we have studied earlier the antiviral activities of two distinct groups of compounds it prompted us to investigate their combined antiviral effect *in vitro*.

Materials and Methods

Viruses and cell cultures. The herpes simplex virus (HSV) type 1 isolated from human conjunctiva in our laboratory and the pseudorabies virus isolated from pig brain were described previously (Cserey-Pechány *et al.*, 1951). HSV-1 was grown in HEp-2 cells. The infectivity was

measured in the same cells by the dilution method in microtitre trays (Linbro, Greiner) and the infective titre was expressed as $TCID_{50}$. The HEp-2 cells were cultured in Eagle's basal medium as modified by Macpherson and Stoker (1962), supplemented with 5% calf serum and 10% tryptose phosphate broth. Pseudorabies virus was propagated in primary chick embryo fibroblast (CEF) cells. CEF cells were maintained in Gey's solution, containing 4% Tris-HCl buffer pH 7.6, 5% calf serum and 0.25% lactalbumin hydrolysate. The infectivity of pseudorabies virus was determined in chick embryo fibroblast monolayers by the plaque method.

Chemicals. Quercetin (Merck) and quercitrin (Calbiochem) were commercial products. Stock solutions were prepared in dimethylsulphoxide and further diluted with culture medium. 5-Ethyl-2′-deoxyuridine (EDU) was synthetized at the Central Research Institute for Chemistry of the Hungarian Academy of Sciences; it was dissolved in physiological saline and was diluted further with culture medium.

Antiviral assay of the compounds. The antiviral activities of the compounds were investigated *in vitro* by the yield reduction test. Monolayer cultures of HEp-2 cells were infected with HSV-1 at a multiplicity of 0.02 $TCID_{50}$/cell. After adsorption for 1 hr at 37 °C, the inoculum was removed and cultures were washed twice with Hanks' solution and supplied with Eagle's medium containing the drugs in different concentrations. After incubation for 20 hr the cultures were frozen and thawed and the cell debris was removed by low-speed centrifugation. The virus content of the supernatant was determined in HEp-2 cells by the dilution method and the virus titre was expressed as $TCID_{50}$/0.1 ml. The antiviral effect on pseudorabies virus was tested in CEF cells using the yield reduction assay. Confluent cell cultures were infected with pseudorabies virus at a multiplicity of 0.0002 PFU/cell. After adsorption for 1 hr at 37 °C, the inoculum was removed and the cultures were washed twice with Hanks' solution. The culture medium with drugs was added and the incubation was continued for 20 hr at 37 °C; then the cells were frozen, thawed and centrifuged. The supernatant was titrated by the plaque method in CEF cells and the virus titre was expressed as $\log_{10}$ PFU/0.1 ml.

Control cultures were treated with media without drugs.

Evaluation of combined antiviral effects of the compounds. The effects of different concentrations of the individual compounds were first investigated to determine the minimal inhibitory concentrations (MIC). The concentration of the compound which alone or in combination caused 1 log inhibition in the virus yield was considered the MIC value. When necessary, these concentrations were calculated by a linear regression method. To investigate the interaction of the two compounds, we applied the single effective concentration of quercetin or quercitrin with varying concentrations of EDU. In another experiment, quercetin or quercitrin were used at different concentrations with varying concentrations of EDU. For evaluation of the drug combinations, the mathematical technique was applied introduced by Allen *et al.* (1983). The fractional inhibitory concentration (FIC) was calculated via the following formula: MIC of drug in combination/MIC of drug alone. In the case of two drugs (A and B), the sum of the FIC values resulted in the FIC index:

$$\frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

FIC indices may be utilized for the identification of drug interactions. If the FIC index < 1, the interaction is synergic; if the FIC index ≈ 1, the effect is additive; if the FIC index is $1.1-1.9$, the effect is indifferent or partially antagonistic; and when the FIC value is > 2, the interaction is antagonistic.

The effects of compounds on the cell cultures. The toxicity of the drugs was investigated also in uninfected cells. Monolayers of CEF cells and HEp-2 cells were treated with culture medium containing the compounds at different concentrations ($1-500$ μmol/l) for 24 hr at 37 °C. The cell cultures were then investigated by microscopical examination, and the cell number was determined after trypsinization and compared to that for the untreated cells.

Results

Toxicity of the compounds

Before investigating the antiviral effects of the compounds, their toxic effects were determined. No visible alteration in cell morphology was

observed by microscopic examination at the applied concentrations, and no decrease was found in the cell number after treatment with the drugs for 24 hr.

Combined effects of quercetin and quercitrin with EDU on HSV-1

In our earlier experiments quercetin and quercitrin caused inhibition of HSV-1 multiplication. Quercetin at a concentration of 250 μmol/l and quer-

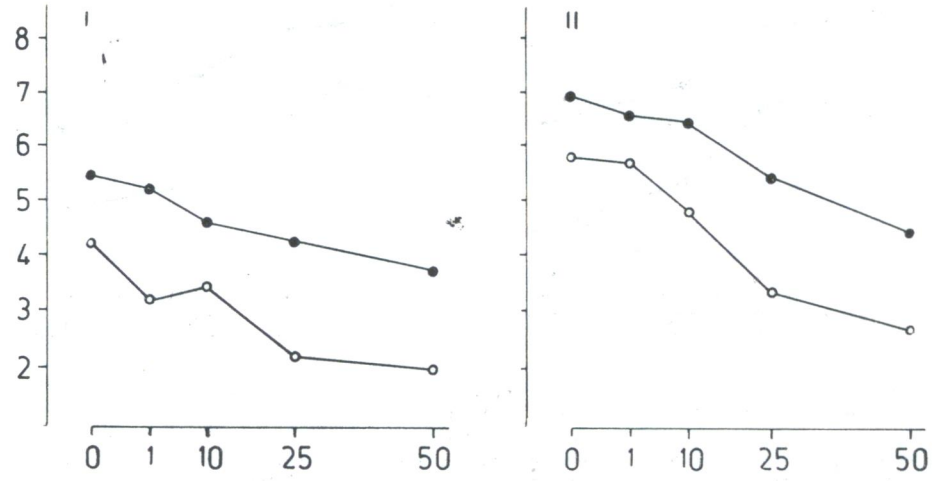

Fig. 1.
Combined antiviral effect of quercetin and EDU (1-I) and of quercitrin and EDU (1-II) on the yield of HSV-1
250 μmol/l quercetin was combined with different concentrations of EDU (mean values o two experiments).
Quercitrin at a concentration of 500 μmol/l was combined with different concentrations of EDU.
Abscissae: EDU concentration (μmol/l); ordinates: virus titre in $\log_{10}$ $TCID_{50}$/0.1 ml
●———● EDU only
○———○ EDU with quercetin (1-I) or quercitrin (1-II)

citrin at a concentration of 500 μmol/l, caused about a 1.5—2 log inhibition in virus yield. EDU at a concentrations of 50 μmol/l caused a similar inhibition. When the effective concentration of quercetin or quercitrin were combined with different doses of EDU, in combination the antiviral activities of the compounds were enhanced (Figs. I-I, II). In another experiment, quercetin or quercitrin at different concentrations were added with varying concentrations of EDU to virus-inoculated cell cultures. In the combinations of quercetin and EDU, the FIC indices were 1.06—1.39. The most effective combination was 100 μmol/l quercetin and 5.7 μmol/l EDU for which the FIC index was 1.06, i.e. it was equivalent to an additive effect. In the combinations of quercitrin and EDU the FIC indices were 1.5—1.59, which meant an indifferent interaction only.

Combined effects of quercetin and quercitrin with EDU on pseudorabies virus

In these experiments the effective concentrations of quercetin (250 μmol/l) or quercitrin (500 μmol/l) were combined with different concentrations of EDU. The antiviral effects of both quercetin and quercitrin were enhanced when applied with EDU (Figs 2-I, II). Quercetin or quercitrin at different con-

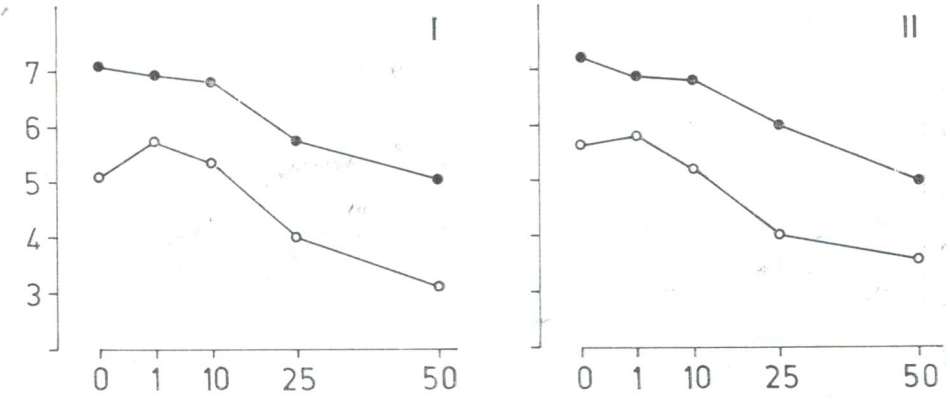

Fig. 2.
Combined antiviral effect of quercetin and EDU (2-I) and quercitrin and EDU (2-II) on the yield of pseudorabies virus
250 μmol/l quercetin was combined with varying concentrations of EDU.
500 μmol/l quercitrin was combined with different concentrations of EDU (mean values of two experiments)
Abscissa: EDU concentration (μmol/l); ordinate: virus titre in $\log_{10}$ PFU/0.1 ml
●———● EDU only
○———○ EDU with quercetin (2-I) or quercitrin (2-II)

centrations were also combined with varying concentrations of EDU. The FIC indices in the combinations of quercetin and EDU were 1.04—1.32. The interaction of 150 μmol/l quercetin and 4.7 μmol/l EDU was most effective, when the FIC index was 1.04, i.e. an additive effect was observed. The lowest FIC indices were obtained when quercitrin was combined with EDU, i.e. 0.78—1.1. The combination of 100 μmol/l quercitrin and 10 μmol/l EDU exhibited synergy, since the FIC index was 0.78.

Discussion

The small number of potent antiviral drugs with specific antiviral effect applicable for human therapy led to the idea that in combinations the already known substances would exert effects with lower non-toxic concentration; this would also decrease the possibility of the emergence of drug-resistant viruses occurring with the use of nucleoside analogues.

The flavonoids are widespread in nature and some of them are used in the treatment of different human diseases (Havsteen, 1983). We described that

quercetin and quercitrin inhibited the replication of herpesviruses in cell culture (Béládi *et al.*, 1981). EDU is one of the potent nucleoside analogues, diminishes the HSV yield in cell culture and its inhibitory effect is dependent on the virus-induced thymidine kinase (De Clercq *et al.*, 1980). The mode of antiviral action of the flavonoids is not yet known. It has been reported that quercetin raises the cAMP level in Ehrlich ascites tumour cells (Graziani and Chayoth, 1977), and also that flavonoids inhibit cAMP phosphodiesterase, which is responsible for the breakdown of cAMP (Beretz *et al.*, 1977; Ferrel *et al.*, 1979). In addition, it has been shown that other cAMP-enhancers, such as dibutyryl cAMP, inhibit the multiplication of HSV-1 and HSV-2 (Stanwick *et al.*, 1979). In addition, has been shown that other cAMP-enhancers, such is possible that the same mechanism acts for antiviral activity of flavonoids. Our data revealed that the combined administration of flavonoids and EDU resulted mainly in an additive antiviral effect, but a synergic effect was obtained with the combination of quercitrin and EDU against pseudorabies virus.

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