Synergism between the compound Dichloroflavan and Chalcone Against poliovirus *in vitro*

التاثيرالتازرى بين المركبين دايكلوروفلافين والجالكون لتثبيط نمو فايروس شلل الاطفال فى المختبر

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Abstract:

Flavone originally founded in some herbs and plants such as beans, tomato and grapefruit. Dichloraflavan (DCF) and the compound chalcone relatively non-toxic, we find in this study that the toxicity of the two compounds to RD and L20B cells ranging between (16-32)µg/ml when they used alone. The minimal inhibitory concentration (MIC) of chalcone was 0.06 µg/ml while the MIC of DCF was 0.03 µg/ml to inhibit (100TCID50) of poliovirus in RD cells and L20B cells. Binary combination of these two compounds showed synergistic activity. We used the Therapeutic Index (TI) to determine the safety of the compound the TI which is the ratio between the toxic concentrations of the drug over the MIC. If the TI one or less means that the compound not advise to use due to the side effect while if the TI more than one compound may be used for treatment, And the fractional inhibitory concentration (FIC) index to determined the synergy between the two compounds

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

The interpretation of the index is as follows: <0.5; significant synergism; 0.5-0.9; suggestive of synergism; =1; effects are additive; 1.1-1.9 partial antagonism; .2 antagonisms.

المستخلص

ماده الفلافون تتواجد فى بعض الاعشاب والنباتات مثل الفاصوليا والطماطه والكريب فروت . ان ماد ة الدايكلوروفالفين و الجالكون غير سامه نسبيا ففي هذه الدراسه عندما تمت دراسه تاثيراتها السمية على خلايا الزرع النسيجي المستعمله وهي RD and L20B , فقد وجدنا ان التراكيزالسامه لماده الدايكلورفلافين تتراوح بين(16-32) µg /ml بينما (0.06) µg /ml يمنع نمو (100TCID50) لفايروس شلل الاطفال في خلايا الزرع النسيجي RD and L20B عندما استخدمت كل ماده منفرده تم استخدام الدليل العلاجي في هذه الدراسه Therapeutic Index (TI) وهو . عباره عن نسبه التركيز السمي على الخلايا مقسوما على اقل تركيزللماده الذي يمنع نمو الفايروس . فأذا كان الرقم 1 فما دون تكون الماده غير صالحه للاستخدام واذا كان هذا الرقم كبير فيعني ان هذا المركب قليل او عديم التاثيرات فى حالة استخدام المركبين معا وبتراكيز تبدا من ضعف التركيز المثبط لكلا المركبين فقد وجدنا ان لهاتين المادتين تفاعل تازرى واضح بدلاله اس تخدام دليل FIC index والموضح في الخلاصه الانجليزيه .

Introduction

The antiviral Chalcone and the compound Dichloroflavan have been studied recently against poliovirus; rubella virus and rhino virus in vitro [1,2,3]

Nevertheless the flavonoids compound have the ability to across the blood-brain barrier; [4] Flavone originally founded in herbs and plants such as beans, tomato and grapefruit [5, 6,7].

Flavonoid prepared by dissolving plant extract powder in ratio 1:5 in methanol and by the using Soxhiet apparatus for 6 hours at (40-60)°C then the solvent removed by using rotary evaporator at 40°C and the crude solid extract was kept in refrigerator until use.

The research on antiviral agent start in 1951 by the discovery of thiosermicarbazones and their effect against vaccine virus infection. However, the story of real and active antiviral agents began in 1962 with the introduction of iodoxuridine (IUDR) [8]. The last few decades have seen dramatic advances in the range and effectiveness of antibiotics and chemotherapeutic agents for treatment of bacterial infection. The development of drugs with effective antiviral activity has proved much more difficult .the root of the problem lies in the nature of viruses and the way in which they damage the cells [9]. There is another problem which is that symptoms of disease usually occur only after substantial virus replication within cells has already occurred. Such cells are damaged or destroyed by the invading virus, and symptoms occurred as a result of this damage.

Antiviral treatment at this stage is ineffective and the majority of infections unnecessary, for by this time the host's own immune defenses have been primed to limit further spread of virus, and recovery follows as damaged cells regenerate.

To be effective, therefore, treatment must be instituted early in the infection, or be given prophylacticaly [10].

Under these circumstances the identity of the invading virus is not usually known, only compounds with broad – spectrum antiviral activity and low toxicity are appropriate. Furthermore, the cost of developing a new chemotherapeutic agent has increased enormously, since many agencies alarmed by the thalidomide (a sedative drug found in 1961 to have caused malformation of the limbs in babies whose mother took it during pregnancy) disaster, Began in 1962 to demand a more comprehensive evidence of lack of teratogenicity and carcinogenicity as well as toxigenicity in man.

For these reasons, antiviral therapy for majority of virus infections is not feasible, nor in many instances it desirable [11].

It has been known for many years that the synergy exhibited by certain combinations of antibacterial drugs in vitro can also be demonstrated in animals and patients. However; there has little work of this sort with antiviral. We have now in this study test for synergy between the compounds Chalcone and Dichloroflavan (DCF) against poliovirus and also checked the toxicity of the combined drugs with the object of selecting a potent for synergy between the compounds Chalcone and Dichloroflavan non-toxic combination for further study in man.

Material and methods

Cells

RD cells were grown at 37ºC in Dulbecco's modified minimal essential medium Egles (BME) supplemented with 10% fetal calf serum (FCS).

L20B cells were grown at 37ºC in BME supplemented with 10% FCS to all media during culture the following agent added (penicillin, Streptomycin and glutamine).The RD cells and L20B supplied by central public health laboratory (Baghdad).

Polioviruses

Laboratory passage strain of poliovirus (originally isolated from stool samples of vaccinated children with poliovirus vaccine); grown in L20B cells monolayer maintained in BME supplemented with 2% FCS. Cultures were harvested at full cytopathic effect (CPE); frozen and thawed; clarified by centrifugation and the supernatant was titrated and it was $6 * 10^{8}$ and then stored at -70ºC.

Antiviral agent

Dichloroflven (DCF) was supplied by Wellcome Research Laboratory, Kent, UK.

The drug obtained in powder form and dissolved in dimethyle sulphoxide (DMSO) (Sigma chemical) then stock solution were stored at 4ºC maintained in EME supplemented with 2% FBS.

The compound chalcone prepared and stored as in DCF

The two compounds were prepared as stock solutions of 10 µg/ml in (DMSO) and stored at 4ºC.

Methods of detecting synergy

Chalcone and DCF were first tested individually to determine their minimal inhibitory concentrations (MIC) and their toxicity to the cell culture. Two fold serial dilutions of on drug starting at 2MIC were made and added in unit volume to rows of wells of micro titer plates containing confluent monolayers of cells. Similar dilutions of second drug were added to the columns of wells in order to produce all possible combinations within the chosen range of concentrations.

To each well was added (100TCID50) of polio virus. The plates were incubated at 33ºC. The end point was complete prevention of cytopathic effect (CPE) in all wells. All experiments were run in triplicate including cell controls.

The toxicity tests for drug combinations run exactly like the above preparation but without the addition of poliovirus.

Results

Studies in tissue culture toxicity

The toxicity of the compound DCF was assessed in RD cells and L20B cells, by inspection of monolayers maintained for 5 days in media with various concentrations of the compound. In RD cells 16 µg/ml Table (1). Induced morphological changes or cell death, but the effect of the compound on L20B is 32µg/ml.

The toxicity of the compound Chalcone was assessed in RD cells and L20B cells, by inspection of monolayers maintained for 5 days in media with various concentrations of the compound. In bough RD cells and L20B 32 µg/ml Table (2). Induced morphological changes or cell death.

Inhibition of CPE by the DCF

Serial 2-fold dilutions of the compound were made starting just below the toxic concentration. These were added with virus to the wells of 96 well microtitre plates containing confluent monolayers of RD cells or L20B cells. They were observed for

CPE daily for 5 days. The minimal inhibitory concentration (MIC) of the drug was calculated according to Karber as 50% end-point. The MIC of the compound were 0.6 µg/ml against poliovirus in both cell culture the RD, and the L20B cells, Table (1)

Inhibition of CPE by the compound chalcone

The test done as described for DCF. MIC of the compound were 0.06 µg/ml against poliovirus in both cell culture the RD, and the L20B cells, virus Table (2)

Table (1): Minimal Inhibitory Concentration (MIC) and Minimal Toxic Concentrations (MTC) (µg/ml) of the Dichloroflven in different cell systems against poliovirus

Cell system	MIC	MTC*	TI**
RD	**0.03**	**16**	**54**
L20B	**0.03**	**32**	**108**

***For the toxicity test the drug concentrations added to the cells without virus**

Table (2): Minimal Inhibitory Concentration (MIC) and Minimal Toxic Concentrations (MTC) (µg/ml) of the Chalcone in different cell systems against poliovirus

Cell system	MIC	MTC*	TI **
RD	0.06	32	54
L20B	0.06	32	54

***For the toxicity test the drug concentrations added to the cells without virus**

$$\text{**Therapeutic index (T.I.)} = \frac{\text{Minimum dose that is toxic to cell}}{\text{Minimum dose that inhibit the virus replication.}}$$

Determination of antiviral synergy

Combination of chalcone and DCF were next tested. As described in Material and methods we used chequerboard titrations with 2-fold dilutions of drugs starting from 2 MIC Table (3) and CPE as end-points. Combination of the two compounds showed evidence of synergy according to the use of fractional inhibitory concentration (FIC) index which calculated as follows [12]

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

The interpretation of the index is as follows: <0.5; significant synergism; 0.5-0.9; suggestive of synergism; =1; effects are additive; 1.1-1.9 partial antagonism; .2 antagonisms.

Table (3): Synergy between Chalcone and Dichloroflven against poliovirus using RD & L20B cells for cultivation

	MIC Of drug in combination µg/ml		FIC index
	chalcone	DCF	
Chalcone	**0.06**	**0.007**	**0.4**
DCF	**0.007**	**0.03**	**0.4**

Discussion

In this study; we identified the toxicity and the antiviral activity of the compounds chalcone and dichloroflven. The toxicity of each compound was assayed in RD cells and L20B cells it were found that concentration of (16-32) µg/ml and over induced morphological changes or cell death. The toxicity of drugs in combination was no great than that of same drugs separately; while the antiviral activity of the compounds

against poliovirus was 0.03µg/ml for DCF and for chalcone was 0.06µg/ml; indeed when we use the TI both drugs seems to be non toxic when used as an antiviral in man (If the TI one or less means that the compound not advise to use due to the side effect while if the TI more than one compound may be used for treatment).

A simple theory of the effect of drug combinations on drug synergy suggests that those drugs with identical modes of action will have an effect which at the most will be additive whine use in combination; whereas those with different modes of action could exhibit synergism. It appears that DCF and chalcone are similar chemically (booth compound belong to flavonoids group) and act on viral peptide synthesis [13, 14]. But; in spite of this; some synergy can be detected; possibly this is because they act differently in detail; i.e. their precise mode or site of action is different although the end result is the same; perhaps they act on different parts of the peptide molecules or at different stages of replication.

The results presented here that the combination of the chalcone and DCF shooed a significant synergism by using the FIC index :(< 0.5; significant synergism; 0.5-0.9; suggestive of synergism; =1; effects are additive; 1.1-1.9 partial antagonism; .2 antagonisms).

Although; there is no evidence that the toxic effects of the compounds on cells are altered by combining them; so; in effect; the therapeutic ratio of each drug is enhanced to the same degree as its antiviral activity (TI become more than 700)

The time of addition and removal each drug recommended giving more explanations for drug action against the virus.

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