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Antiviral Activity of Enviroxime Against Polio Virus and Rubella Virus in Tissue Culture

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Abstract
Enviroxime is an antiviral compound that inhibits the replication of polio and rubella virus. The antiviral activity of this compound was tested in two cell lines (L2OB and RD for poliovirus and HeLa and WISH for Rubellavirus). At a concentration of 32μg/ml or less, the compound is relatively non-toxic to cell lines but has significant antiviral effect. We found the minimal inhibitory concentration (MIC) of 0.06 μg/ml for polio, and 0.125 μg/ml for rubella virus. The therapeutic index (TI) defined as the ratio of the minimal dose of the drug that is toxic to the cells to the dose which inhibit the viral multiplication, is used to evaluate the drug activity. If this index is more than one, the margin of safety for the drug is large. In this study the TI of enviroxime against poliovirus was 54 while it was 14 against Rubella virus. Hence we speculate that this compound if used as an antiviral agent in humans, would have minimal or no side effect.

Keyword: Poliovirus, Rubellavirus, Antiviral Therapy, Enviroxime

Introduction

The rubella virus is a member of the genus Rubivirus (an enveloped virus, negative sense single stranded RNA) (1). Rubella is a mild disease that affects children and adolescents worldwide and may also affect young adults. However, the consequences are devastating for women early in pregnancy as infection to the fetus may result in birth defects. An accurate diagnosis becomes critical in pregnancy (1). On the other hand, poliovirus infection is either asymptomatic or causes a mild, undifferentiated febrile illness in majority of patients. Spinal and bulbar poliomyelitis occurs rarely. The paralytic poliomyelitis is not always preceded by minor illness and is usually irreversible. Central nervous poliomyelitis has largely been eliminated by immunization. This virus infects the gray matter of the spinal cord, brain stem and cortex, after invading the anterior horn cells in the lumbar segments. The death occurs due to respiratory paralysis. If muscle weakness due to poliovirus infection

shows no signs of recovery, the chance of regaining any meaningful function is minimal (2 &3).

Symptoms of disease manifest after a substantial virus replication as the invading virus destroys the cells. Therefore, the antiviral treatment at this stage is ineffective and unnecessary. The recovery follows and damaged cells regenerate once the host's immune system limit further spread of the virus. Therefore, to be effective a treatment must be instituted early during the disease course or be given prophylactically even when the identity of the invading virus is not known (4). Compounds with broad-spectrum antiviral activity and low toxicity are appropriate (5).
In this study, we aimed to examine the activity of an antiviral compound called "enviroxime" against two different RNA viruses (Rubella & Poliovirus).

Materials and Methods

Cells Lines
WISH cells were grown at 37º C in MEM (Gibco) supplemented with 10% fetal bovine serum (FBS). HeLa cells were grown at 37 º C in MEM supplemented with 10% FBS. RD cells (human rhabdomyosarcoma) were grown at 37 º C in Eagle minimum essential (EME) supplemented with 10% fetal calf serum (FCS). L20B cells were grown at 37 º C in EME supplemented with 10% FCS. To all cell cultures the following agent were added (1% glutamine, 100 unit/ml penicillin and 0.1 mg/ml. Streptomycin). All cell cultures were supplemented with 2% CO_2 during incubation. The concentrations of cells / ml were 10^4

Viruses
Two strains of viruses were used. Laboratory passed strain of poliovirus grown in L20B cells monolayer maintained in EME supplemented with 2% FBS. Cultures were harvested at full cytopathic effect (CPE), frozen and thawed, clarified by centrifugation and the suspension was stored at -70 º C. Laboratory passed strain of rubella virus grown in chick embryo fibroblast cells monolayer maintained in MEM supplemented with 2% FCS Cultures were harvested at full cytopathic effect (CPE), frozen and thawed, clarified by centrifugation and the suspension was stored at -70 º C.

The antiviral agent
Enviroxime was prepared at the Lilly research laboratory, as an equal mixture of syn- and anti-isomers. The drug, obtained in powder form, was prepared as stock solution 500 μg/ml in dimethyle sulfoxide (DMSO). (Sigma chemical) then stock solution was stored at 4 º C.

The determination of therapeutic index
Therapeutic index (TI) of a drug is ratio of its minimum toxic dose (MTD) to its minimum effective dose (MED):

TI = MTD/MED

If this index is one or less, the drug has significant side effects, but if the index is larger than one, the margin of safety is large. Methods of detecting antiviral activity **of** enviroxime and DMSO were first tested individually to determine their toxicity and the antiviral activity. In case of DMSO only the concentration of 1/10 in EME was used. Twofold serial dilutions of Enviroxime , starting from 50 μg/ml for the toxicity test and ½ toxic concentration of enviroxime, were used for the determination of MIC of the compound.

Results
Studies in tissue culture toxicity
The toxicity of the enviroxime was assessed in HeLa cells, WISH cells, RD cells and L20B cells, by inspection of monolayers maintained for 5 days in media with various concentrations of the compound. In HeLa cells, WISH cells and RD cells 16μg/ml concentration induced the morphological changes or cell death, but at 32μg/ml, no cytotoxic antiviral effect of DMSO was noted on L20B cells and virus.

Inhibition of CPE by the enviroxime
Serial 2-fold dilutions of the compound were made starting just below the toxic concentration. These were added with the virus to the wells f 96 well microtitre plates containing confluent monolayers of HeLa cells, WISH cells, 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 was 0.06 μg/ml against poliovirus in both cell culture the RD, and the L20B cells, while the MIC of enviroxime against rubella virus was 0.125μg/ml (Table 1).

Reduction of plaques by the Enviroxime
We studied the yield of virus in the presence of selective concentrations of the compound agent poliovirus in L20B cells (using 5-cm.petri dishes with confluent monolayer of cells infected with 50 PFU of poliovirus, the EME

Table 1. Minimal inhibitory concentrations (MICs) and minimal toxic concentrations (MTC) (μg/ml) of the enviroxime in different cell systems.

Cell system	Type of virus	MIC	MTC*	TI
HeLa	Rubella	0.125	16	14
WISH	Rubella	0.125	16	14
RD	Polio	0.06	16	28
L20B	Polio	0.06	32	54

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

Table 2. Activity of enviroxime against poliovirus demonstrated by plaque reduction in L20B cells.

Enviroxime μg/ml	No. of plaque	Ratio of plaque formed untreated / treated
0	46	1
0.03	34	1.35
0.06	3	15.3
0.125	0	(not applicable)

All experiments were run triplicate and usually repeated 2-3 times

supplemented with 1% Noble agar) the CPE was observed after staining the residual cells with crystal violet or theCPE were read by microscopy of unstained cell monolayers. The yield was greatly reduced by the enviroxime (Table 2).

Discussion

In spite of relatively extensive efforts to develop antiviral drugs, the efficacy of antiviral compounds can be quantified by using the therapeutic index (6) The present study of enviroxime confirms that this compound is relatively non-toxic with an inhibitory effect against poliovirus in RD cells and L20B and an inhibitory effect against Rubellavirus in HeLa cells & WISH cells. The toxicity of enviroxime to L20B is slightly higher than to other three types of cell cultures, may be due to the fact that these three cell lines are of human origin whereas L20B cell line is of mice origin (7,8).

Since the inhibition of CPE is relatively simple test, it has some value for preliminary experiments to evaluate antiviral drugs. It seemed probable that a more sensitive test based on plaque titration could have given more accurate and reproducible result. Indeed we used this method just to compare the sensitivity of CPE and the plaque titration. We chose poliovirus and L20B because the L20B cells are more sensitive to poliovirus than RD cells (9). However poliovirus plaques were inhibited by nearly the same concentration of enviroxime as was required to inhibit 100 TCDI50 in L20B cells. The L20B cells are more sensitive to poliovirus than RD cells (10).

It is important to bear in mind that the conditions of human experimental studies differ from that done in tissue culture. Bucknal (11) indicated that 70% of compound active against viruses in tissue culture failed to inhibit viral multiplication in organ culture or in animals, therefore test in human organ culture for the detection of antiviral activity of enviroxime against rubella virus and poliovirus, were very important.

Indeed we have done some experiments for a compound called chalcone against rubella virus in tissue culture and in organ culture and we found that the compound act at the same concentration in both system (12). Unfortunately at the time of testing the antiviral activity of enviroxime, we didn't have a human organ culture (such as human embryonic nasal epithelium and human embryonic tracheal culture. The method of preparing organ culture has been previously described by Ahmad and Tyrrell (4). When we use the TI for drug against rubella virus we find that this index is 14 while this index is more than 54 when the drug is used against poliovirus.

The difference between inhibitory concentration of the enviroxime against rubella virus and poliovirus may be due to the differences between the two viruses as one is enveloped and the other is non-enveloped virus. The mechanism of action of enviroxime was proposed as an anti-picornavirus compound that target viral protein 3A & 3AB and suppresses replication steps of picornavirus during its effect on cellular kinase inhibitor (13,14).

Our study confirms that enviroxime is relatively non-toxic with an inhibitory effect against poliovirus and rubella virus in all cell lines. The susceptibility of poliovirus to the compound is demonstrated by inhibition of CPE produced by 100TCID50 in tissue culture of RD cells, and L20B cells. However we found that the sensitivity of virus to the drug varied between different experiment, the variation may be due to differences between batches of cell or may also partly be due to change in the drug concentration as the drug was not prepared freshly each time but prepared from the stock. Furthermore the sensitivity of poliovirus to the drug was less in RD cells than in L20B cells. The virus titration in RD cells and L20B cells were same. This suggests that the lack of inhibition by the drug may have been partly due to behavior of the drug in the different cells, rather than to insusceptibility of the virus to the drug. Therefore enviroxime if used in humans should be first checked in animal models. If human trials suggest low side effects, we recommend developing this compound further.

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