Testing of Varuflu for Antiviral Activity *In Vitro*

Report of the research work ordered and sponsored by Farmacevtska industrija INTER - EVROGENEKS (Novo selo, Strumica, Republic of Macedonia)
and performed in the Institute of Microbiology (Department of Virology), Bulgarian Academy of Sciences (Sofia, Bulgaria)

Head of research team: Prof. Angel S. Galabov, M.D., D.Sc.

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Substances Tested

Varuflu

Dark clear odorless liquid, kept *in vitro fusca* at 4^0C, consigned by …… This initial liquid is further referred as the "starting solution". For the needs of the experiments the starting solution was diluted *ex tempore* in 0,5 lg dilutions in a maintenance medium of Dulbecco's modified Eagles's medium DMEM (Gibco®, Invitrogen Life Technologies) supplemented by 0.5% bovine fetal serum (Gibco®, Invitrogen Life Technologies) , 3.7 mg/mL sodium hydrogen carbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (100 UI/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamycin).

Reference Antivirals

- Guanidine hydrochloride vs. Coxsackievirus B1
- Ribavirin vs. influenza virus B, respiratory syncytial virus(Long) and human adenovirus 2
- Rimantadine hydrochloride vs. influenza virus A(H3N2)
- Acyclovir provided from Burroughg Welcome Co., Research Triangle Park, NC**,** was used as a reference anti-herpesvirus compound.

Viruses

Coxsackievirus B1 1 (Connecticut 5) [CVB1], from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in FL cells (maintenance solution Dulbecco's modified Eagles's medium DMEM (Gibco®, Invitrogen Life Technologies), supplemented by 0.5% bovine fetal serum (Gibco®, Invitrogen Life Technologies), 3.7 mg/ml sodium hydrogen carbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml. Cytopathic effect was recorded microscopically followed by a colorimetric evaluation by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] procedure [Mosman, 1983]. Virus titer was determined according to Reed and Muench [1938]. CVB1 infectious titer: $10^{8.0}$ $CCID_{50}$ /ml, resp. 8.5 x 10^8 PFU/ml.

Influenza A virus [Aichi/2/68 (H3N2)] [IAV]**,** from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria). The stock virus represented alantoic fluids of virus-inoculated 10-days-embryonated eggs, cultivated at 37^o; infectious titer $10^{7.5}$ $CCID_{50}$/ml.

Influenza B virus (Lee/40) [IBV], ATCC No. VR-101 (USA). The stock virus represented alantoic fluids of virus-inoculated 10-days-embryonated eggs, cultivated at 35^o C; infectious titer $10^{7.5}$ $CCID_{50}$/ml.

Respiratory syncytial virus A2 [RSV-A2], kindly supplied by the District Center of Hygiene and Epidemiology, Plovdiv (Bulgaria). The virus was grown in HEp-2 cells (maintenance solution Dulbecco's modified Eagles's medium DMEM (Gibco®, Invitrogen Life Technologies), supplemented by 0.5% bovine fetal serum (Gibco®, Invitrogen Life Technologies), 3.7 mg/ml sodium hydrogen carbonate, 10 mM HEPES buffer (AppliChem

GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). Infectious titer $10^{4.5}$ $CCID_{50}$/ml.

Human adenovirus type 5 [HAdV5], kindly supplied by District Center of Hygiene and Epidemiology, Plovdiv (Bulgaria). The virus was grown in FL cells (maintenance solution Dulbecco's modified Eagles's medium DMEM (Gibco®, Invitrogen Life Technologies), supplemented by 0.5% bovine fetal serum (Gibco®, Invitrogen Life Technologies), 3.7 mg/ml sodium hydrogen carbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). Infectious titer $10^{6.5}$ $CCID_{50}$/ml.

Bovine viral diarrhea virus (TVM strain) [BVDV], cultivated in calf trachea cell line [CT cells] (maintenance solution DMEM Gibco BRL, Paisley, Scotland, UK, plus 0.5% fetal bovine serum Gibco BRL, Scotland, UK); infectious titer $10^{7.0}$ $CCID_{50}$/ml.

Herpes simplex virus type 1 (strain Victoria) [HSV-1] was received from Prof. S. Dundarov, National Center of Infectious and Parasitic Diseases, Sofia. The virus was cultivated in cell culture of Madin–Darby bovine kidney (MDBK) (maintenance solution DMEM Gibco BRL, Paisley, Scotland, UK, plus 0.5% fetal bovine serum Gibco BRL, Scotland, UK). The stock virus infectious titer was 10^7 $CCID_{50}$/ml.

Cells and Media

FL cells (collection of the Stephan Angeloff Institute of Microbiology, BAS, Sofia, Bulgaria) were routinely sub-cultured once or twice weekly in a growth medium consisting of Dulbecco's modified Eagles's medium DMEM (Gibco®, Invitrogen Life Technologies), supplemented by 5% bovine fetal serum (Gibco®, Invitrogen Life Technologies), 3.7 mg/ml sodium hydrogen carbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). Cells are incubated at 37^0C in a humidified atmosphere with 5% CO_2 and were employed for CV B1 cultivation and testing.

HEp-2 cells (National Bank for Industrial Microorganisms and Cell Cultures, No. NBIMCC-95, Sofia, Bulgaria) were grown in medium consisting of Dulbecco's modified Eagles's medium DMEM (Gibco®, Invitrogen Life Technologies), supplemented by 5% bovine fetal serum (Gibco®, Invitrogen Life Technologies), 3.7 mg/ml sodium hydrogen carbonate, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). Cells are incubated at 37^0C in a humidified atmosphere with 5% CO_2. They were used for cultivation and experiments with RSV and AdV2.

MDCK (Madin-Darby canine kidney) cells (NBL-2) (ATCC No. CCL-34, USA) were grown in medium containing 10% fetal calf serum in DMEM Gibco BRL, USA, supplemented with 10 mmol/l HEPES buffer (Gibco BRL, USA) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). The cells were employed for experiments with IAV and IBV.

CT cells (calf trachea cell line) from the cell culture bank of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), were cultivated in Costar plastic vessels

(USA) with DMEM Gibco BRL, containing 10 % fetal bovine serum (Gibco) and anibiotics (penicillin 100 U/ml, and streptomycin, 100μg/ml).

MDBK (Madin-Darby bovine kidney) cells (National Bank for Industrial Microorganisms and Cell Cultures, Sofia) were grown in DMEM medium containing 10% fetal bovine serum Gibco BRL, USA, supplemented with 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in CO_2 incubator (HERA cell 150, Heraeus, Germany) at 37°C/5% CO_2. The cells were employed for experiments with HSV-1.

Methods

Determination of Cytotoxicity

Monolayer cells in 96-well plates are inoculated with 0.1 mL of the tested solution in several serial dilutions performed in a maintenance medium. Cells inoculated with 0.1 mL maintenance medium (no compound in the medium), serve as a control. Each tested dilution is inoculated in 6 wells of the cell culture plate. Then cells are incubated at 37^0C in a humidified atmosphere with 5% CO_2 and read microscopically on the 24th and the 48th hour post inoculation. The maximal tolerated concentration (MTC) is determined as that concentration at which no visible changes in the cell monolayer are observed.

The MTT procedure is performed on the 24th and the 48th hour, too. The optical density (OD) is measured with a spectrophotometer at 540 nm, with 690 nm as a reference read-out. The 50% cytotoxic concentration (CC_{50}) is calculated in comparison to the cell control by applying the regression analysis with the help of Origin 6.1 computer program.

Antiviral Activity Testing

The cytopathic effect (CPE) inhibition test is used for measuring the antiviral effect. Monolayer cells in 96-well plates are inoculated with 0.1 mL virus suspension containing 100 $CCID_{50}$. After an hour for virus adsorption (two hours in the case of HRSV-A2) in a humidified atmosphere at 37^0C and 5% CO_2, excessive virus is discarded and cells are inoculated with 0.2 mL of maintenance medium containing serial 0.5 lg dilutions of the tested preparation. Mock-infected cells are left for cell and toxicity controls. The virus CPE is scored daily by inverted light microscope (Olympus CK40, Japan) at 125x and 400x magnification on a 0-4 basis (4 representing total cell destruction) till the appearance of its maximum in the virus control wells (with no compound in the maintenance medium) – the 48th hour p.i. for CV-B1, and the 5th day (120th hour) p.i. for HAdV-5 and HRSV-A2. When maximum CPE in the virus control wells is reached, cells are processed according to the MTT procedure described above. The percent of virus CPE protection is calculated by the following formula [Pannecouque et al., 2008]:

$$\frac{mean\,OD_{Test} - mean\,OD_{VC}}{meanOD_{TC} - meanOD_{VC}}\,x\,100$$

$(OD_{Test} - OD_{VC})/(OD_{TC} - OD_{VC})$ x 100 (%), where OD_{Test} is the mean optical density (OD) of the test sample, OD_{VC} – the absorbance of the virus-infected control (no compound in the maintenance medium), and OD_{TC} – the OD of the mock-infected control (toxicity control).
The 50% virus inhibitory concentration (IC_{50}) is determined by applying the regression analysis with the help of Origin 6.1 computer program and it is expressed as the concentration that achieves 50% protection of virus-infected cells.

The selectivity index (SI) is determined as the ratio between CC_{50} and IC_{50} (SI = CC_{50}/IC_{50}).

Results

Cytotoxicity of Varuflu

Cytotoxicity of Varuflu to all cell cultures used in the antiviral screening

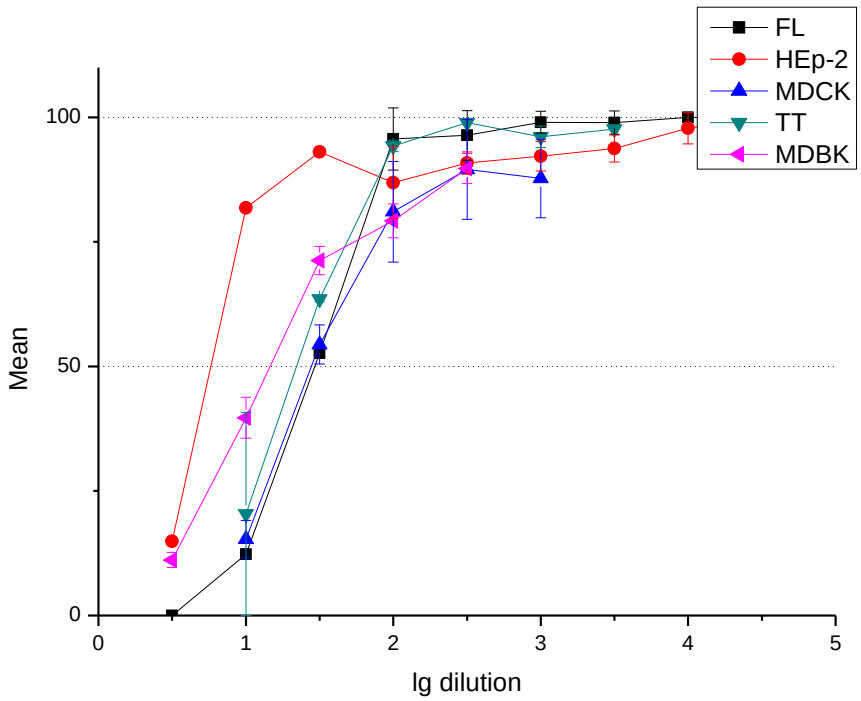

Fig. 1. Cytotoxicity of Varuflu to the cell cultures used in the antiviral screening: FL, Hep-2, MDCK, TT and MDBK

The starting solution of Varuflu is toxic to the monolayer FL and HEp-2 cultures .

Cytotoxicity to FL cells

Visible changes in the morphology of FL cell monolayers due to the cytotoxic effect of the tested preparation are observed at a 100-fold dilution (2lg) during the first 24 hours p.i. During the 48 hour period of observation rather discrete changes could be observed also at a 2.5 lg dilution. No changes are seen at 1000-fold (3 lg) dilution of the starting solution.

Cytotoxicity to FL cells:

MTC = 2.5 lg (dilution 1:320)
CC_{50} = 1.4 lg (dilution 1:25)

Cytotoxicity to HEp-2 cells

The sensitivity of monolayer HEp-2 cells to the cytotoxic effect of Varuflu is similar to that of FL cells. Visible changes in the morphology of the monolayer are observed at 2g dilution. No changes are seen at 3 lg dilution.

Cytotoxicity to HEp-2 cells:

MTC = 2.5 lg (dilution 1:320)
CC_{50} = 1.6 lg (dilution 1:37)

Antiviral activity

Testing against Coxsackievirus B1, human adenovirus 5 and respiratory syncytial virus

The results of the antiviral tests for the activity of Varuflu against the replication of CV-B1, HAdV-5 and HRSV-A2 are presented on Fig. 2.

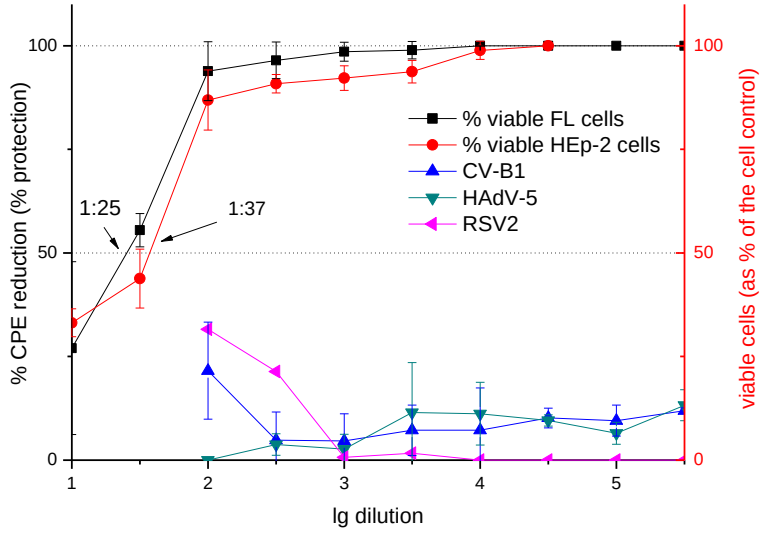

Fig. 2. Effect of Varuflu on the replication of CV-B1, HAdV-5 and HRSV-A2 (dose-response curves). Cytotoxicty of Varuflu to FL and HEp-2 cell monolayer.

Nontoxic dilutions of Varuflu exert very slight and statistically ignorable antiviral effect on the replication of the tested viruses. Varuflu is capable of inhibiting 30% the replication of RSV *in vitro* only at the highest possible nontoxic concentration and only 20% of the replication of CV-B1. The replication in vitro of HAdV-5 is not affected by Varuflu.

Testing against influenza A virus

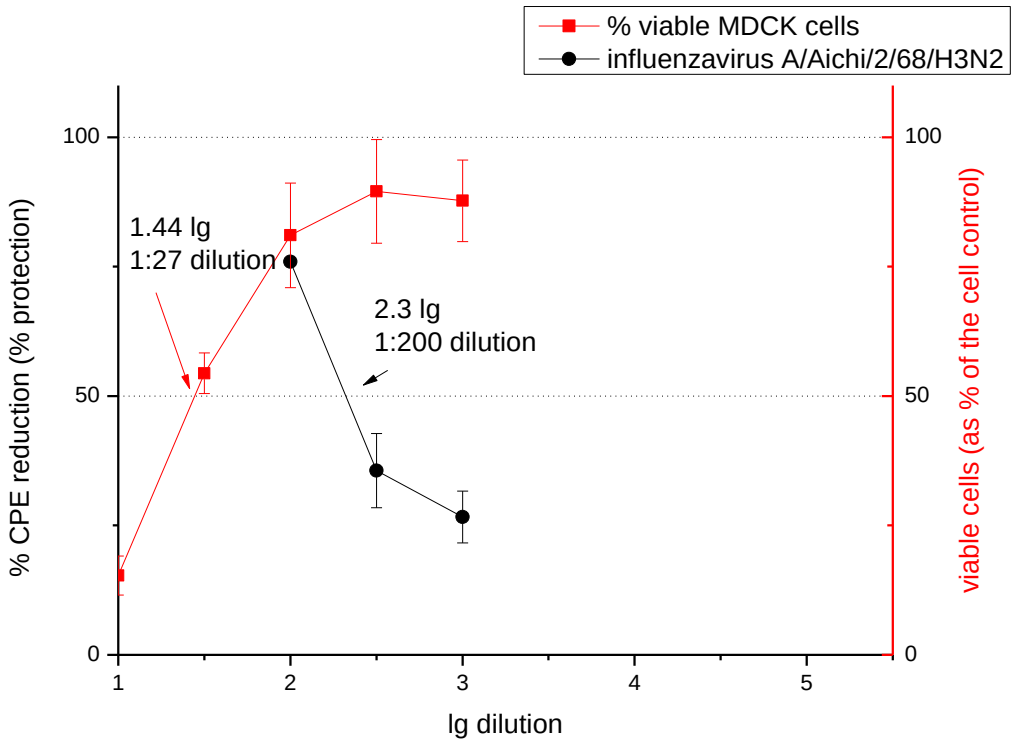

Fig. 3. Effect of Varuflu on the replication of influenza virus A(H3N2) (dose-response curves). Cytotoxicty of Varuflu to MDCK cell monolayer.

Testing against bovine virus diarrhea virus

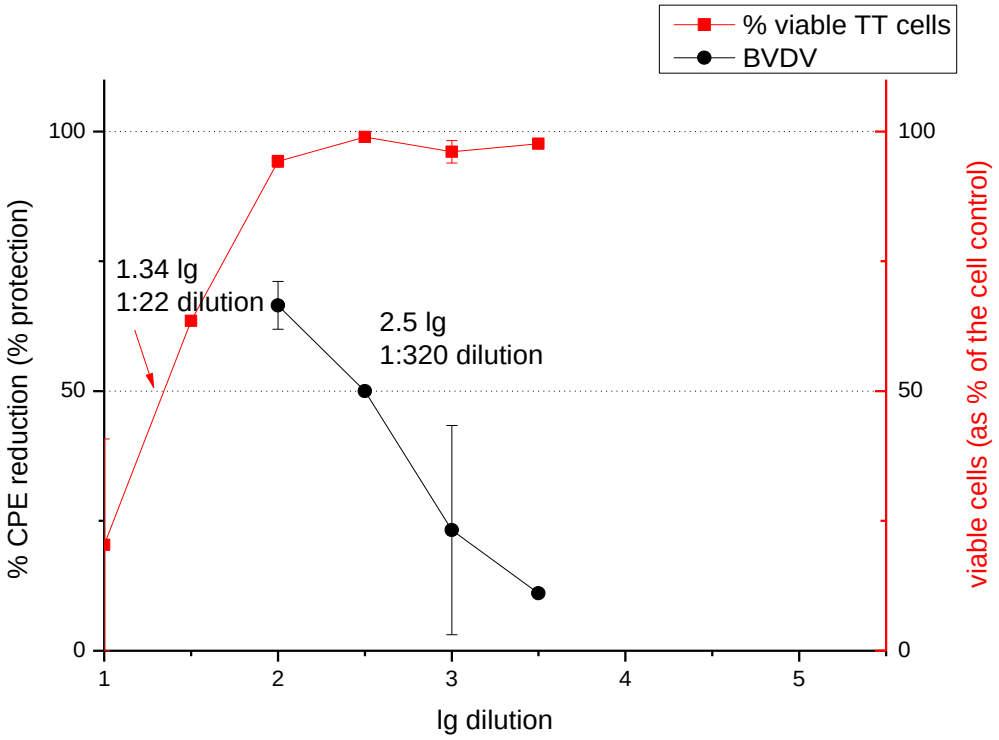

Fig. 4. Effect of Varuflu on the replication of bovine viral diarrhea virus (dose-response curves). Cytotoxicty of Varuflu to TT cell monolayer.

Testing against herpes simplex virus type 1

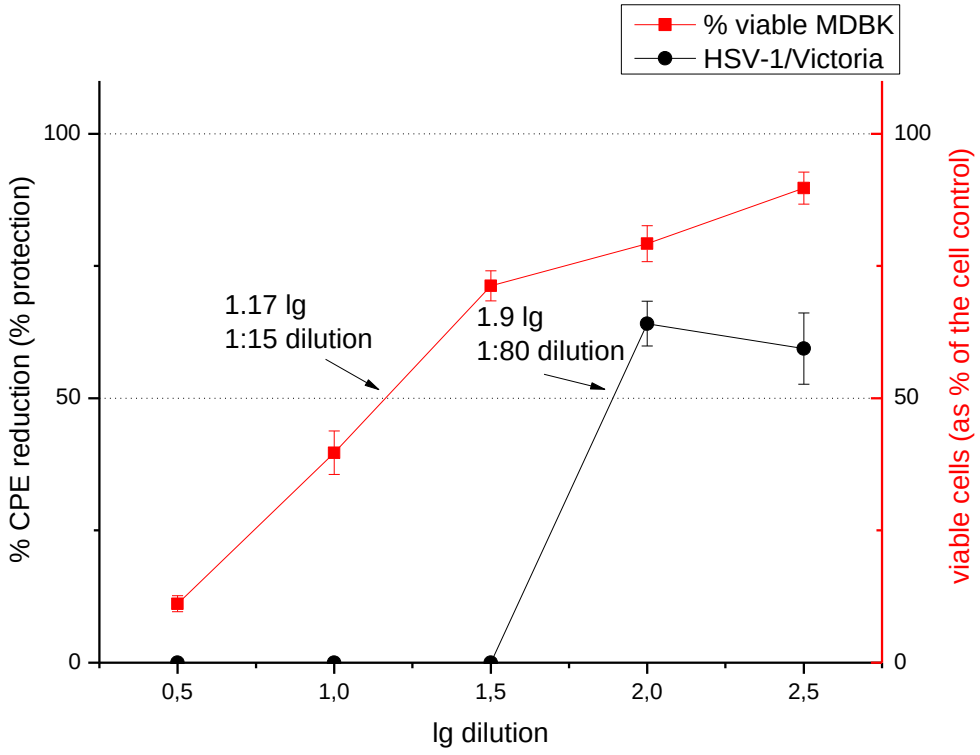

Fig. 5. Effect of Varuflu on the replication of herpes simplex virus type 1 ((dose-response curves). Cytotoxicty of Varuflu to TT cell monolayer.

Table 1. Antiviral effects of Varuflu against influenza virus A(H3N2), bovine viral diarrhea virus and herpes simplex virus 1

Virus tested	Antiviral effect IC_{50}*	Cytotoxicity CC_{50}*	Selectivity index (CC_{50}/ IC_{50}) SI
Influenza A(H2N2)	1:200	1:27	7.4
BVDV	1:320	1:22	14.5
HSV-1	1:80	1:15	5.3

* Dilution of the starting solution of Varuflu

Conclusion

- **Varuflu possess a marked antiviral activity against the replication *in vitro* of the bovine viral diarrhea virus, a surrogate hepatitis C virus.**
- **Varuflu shows a weak antiviral activity against the replication *in vitro* of influenza A virus (A/H3N2) and herpes simplex virus type 1.**
- **Varuflu does not possess antiviral activity against the replication *in vitro* of the tested representatives of *Picornaviridae, Paramyxoviridae* and *Adenoviridae*.**

References

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Reed, L., Muench, H. (1938): A simple method of estimating 50% endpoints. *Amer. J. Hygiene*, **27**, 493-497.

Pannecouque, C., Daelemans, D., De Clercq, E. (2008): Tatrazolium-based colorimetric assay for the detection of HIV replication inhibitors: revisited 20 years later. *Nature Protocols*, **3**, 427-434.