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RESEARCH ARTICLE

Determination of an Antiviral Activity of a Composition Comprising Glutathione Reductase (GSSG-R) and Oxidized Glutathione (GSSG) for Pharmaceutical use: Experiments *In vitro* and *In vivo*

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ABSTRACT

There are several viruses that are not currently covered by efficient antiviral terapies, as towards Poliovirus and the new Covid19. The spread of atypical viral strains strongly raises the need of having efficient terapies with wide coverage against viruses.

In the following experiments it was used a composition comprising Glutathione Reductase (GSSG-r) and Oxidized Glutathione (GSSG), which we call GR compound, for pharmaceutical use as antiviral, in vitro, towards RNA nad DNA virus, and in vivo towards a Swine Flu Virus (H1N1) adapted to the mouse .

Glutathione is present in the cells in form of Reduced Glutathyione (GSH) and Oxidized Glutathyione (GSSG). The GSH:GSSG ratio indicates the antioxidant capacity of the cell and it is maintained in favour of GSH due to GSSG-r, an enzyme belonging to the class of oxidoreductase which rigenerates GSH starting from GSSG.

The GR compound binds to the membrane of cells infected with virus, and repolarizes the membranes so that the Glutamate comes back in the cell and the synthesis of Glutathione starts again. Maintaining a correct GSH:GSSG ratio is an essential requirement for the vitality and survival of the cell. This technology exerts its primary action electrochemically (oxidation-reductive state) both in the host cell and in the virus. Practically the virus, entering the cell, depolarises the cell membrane thus preventing the entry of Glutamate (due to the inhibition of its transport carrier), which is fundamental for the synthesis of Glutathione.

Glutathione continuosly passing from reduced to oxidized generates and maintains the reductive-oxide state, necessary for the cell; all this leads to the non-formation of the sulfur bridges of viral proteins, which thus, changed in allosterism and spatial configuration, are completely eliminated by the immune system; all viruses are weakened, HIV, Covid19, Polio, Ebola, because all viruses must go through this step: the protein formation.

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BACKGROUND OF THE EXPERIMENTS THAT SUPPORT THIS NEW TECHNOLOGY

The therapy of viral pathologies currently uses chemotherapy agents with multiple action mechanisms. In particular, considering flu as a typical viral disease, there are used M2 inhibitors such as Amantadine and neuraminidase inhibitors such as Oseltamivir and Zanamivir. It is known that viruses are capable of acquiring resistance against antiviral agents, hence there strongly arises the need of therapies alternative to those using antiviral agents, also due to the fact that use of these compounds, especially Amantadine, is often associated to severe adverse effects and thus it is not recommended unless under particular conditions [1].

In addition, there are several viruses that are not currently covered by efficient antiviral therapies. By way of example, currently there are no therapies capable of fighting Poliovirus. The people intensive migrations that occur nowadays have also spread viral strains typical of some areas of the world to areas where the same were unknown, in other cases they have brought back problems related to viral strains that had been considered eliminated in that specific area. Thus, there strongly arises the need of having efficient therapies and with wide coverage against virus [2,3].

Glutathione is present in cells in form of Reduced Glutathione GSH and Oxidized Glutathione GSSG. The GSH:GSSG ratio indicates the antioxidant capacity of the cell. Said ratio is maintained in favour of GSH due to GSSG-r, an enzyme belonging to the class of oxidoreductase, which regenerates GSH starting from GSSG, by transferring electrons transferred from the NADPH cofactor, a derivative of vitamin PP (nicotinic acid). Maintaining a correct GSH:GSSG ratio is an essential requirement for the vitality and wellbeing of the cell. A misbalanced GSH:GSSG ratio was observed in numerous pathologic conditions, for example in viral infections, tumours, cystic fibrosis, neurodegenerative diseases. In said pathologic conditions there arises a misbalance in the GSH:GSSG ratio, which is displaced in favour of GSSG hence the cell loses the oxide-reductive balance [4,5].

Administration of GSH was proposed in order to counter such loss of balance. Actually, GSH is the most known and powerful physiological antioxidant, and thus already used as a supplement in anti-aging therapies, and it was surprisingly proven to have antiviral activity [6]. The supplementation of GSH however proved to be complex, in that it is poorly absorbed in the gastrointestinal tract. This drawback was overcome by administering GSH precursors, such as S-Adenosyl-1-Methionine (SAMe) and/or N-Acetylcysteine (NAC).

MECHANISM OF THE TECHNOLOGY

Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals. It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and cysteine. The carboxyl group of the cysteine residue is attached by normal peptide linkage to glycine.

The lack of Glutathione cannot be compensated by its simple intake, because the cells do not adsorb Glutathione but only take Glutamate from the outside to form it.

This technology exerts its primary action electrochemically (oxidation-reductive state) both in the host cell and in the virus. Practically the virus entering the cell depolarises the cell membrane, thus preventing the entry of glutamate (due to the inhibition of its transport carrier), which is fundamental for the synthesis of Glutathione, togheter with cysteine and glycine which are produced inside the cell, unlike glutamate which must be taken from outside, in the human body from the bloodstream: therefore the Glutathione synthesis starts again in the cell and the Glutathione, in its continuous passage from reduced to oxidized form, generates and maintains the reductive oxide state necessary for the cell survival; all this leads to the non-formation of the disulfide bridges of viral proteins, which thus, changed in allosterism and spatial configuration, are completely eliminated by the immune system. This happens for every virus from Ebola, Hiv, Corona, to Polio virus, because all viruses must go through this step. This technology does not kill the viruses but weakens them so much that they are eliminated by the immune system.

More specifically in this technology the compound of GSH and GSSG-r has simply the capacity to establish the correct polarity of the cell membranes,so that the glutamate can enter the cell and the synthesis of Glutathione starts again. The technology is innovative because it is the only one that manages to restart the natural antiviral activity of reduced glutathione, blocked by the entry of the virus, restoring the synthesis of glutathione itself, ensuring the flow of glutamic acid (fundamental to synthesize Glutathione together with cysteine and glycine already present in the cell) from outside (the bloodstream) into the cell ("*In vitro*" experiments needs the addition of glutamate, because it is a "closed system").

The technology is a universal antiviral since the activity of Glutathione is in no way selective of viral species, in fact, the proposed work speaks of a wide-ranging antiviral action that is from DNA to RNA viruses.

It should be noted that among these viruses, the work also mentions an antiviral action against the polio virus which no antiviral, apart from the vaccine, contrasts.

As regards the statistical framework of this technology, it should be noted that the antiviral activity itself is practically explained by the stable presence of reduced Glutathione. This is to say that we are not in presence of a synthetic molecule, but of the correct functionality of the oxide / reduction

system of the host cell; therefore, the efficacy percentage is around 75% for all viruses, this means that if the synthesis of reduced Glutathione is guaranteed, as the technology does, the oxide/reductive system of the host cell, working properly, protects 75% of the cases from viral infections.

MATERIALS AND METHODS

The present experiments regard a composition comprising GSSG-r and GSSG or pharmaceutically acceptable salts thereof for pharmaceutical use as antiviral or antibacterial agents and, more generally, in reducing the cellular damage caused by the loss of the oxide-reductive balance of the cell, such as for example when the cellular environment loses the oxide-reductive balance (reduction of the GSH/GSSG ratio) thereof due to an intracellular viral infection and in the protection of the toxicity of the radicals produced by the radiolysis of cellular water.

Regarding the present experiments, GSH (reduced Glutathione) refers to a tripeptide having the L-Glu-L-Cys-Gly structure, while oxidized Glutathione GSSG refers to a dipeptide in which two molecules of GSH are bonded to each other through a disulphide bond.

A particularly important aspect lies in the fact that said composition has a wide spectrum activity, being efficient towards RNA or DNA virus, with or without viral envelope, retrovirus and bacteria, at non-cytotoxic concentrations.

Preparation and Storage of the GSSG-r + GSSG formulation, that we will call GR compound. The composition of the present invention comprises GSSG-r and GSSG or a pharmaceutically acceptable salt thereof, combined together for example according to the method claimed herein. The starting GSSG-r can be lyophilized GSSG-r, obtained from human erythrocytes, GSSGr in suspension with ammonium sulphate or recombinant human GSSG-r. In a further embodiment, GSSG-r is conjugated with pharmaceutically acceptable molecules, for example pegylated GSSG-r is used [7].

GSSG can be obtained through any of the conventional methods used for preparation thereof. For example, GSSG can be obtained by chemical synthesis through the procedure described in Bull Chem Soc Jpn. 1980; 53.

In case of use of GSSG-r in suspension with ammonium sulphate, it is recommendable to eliminate ammonium sulphate due to the toxicity thereof.

Ammonium sulphate can be eliminated for example through dialysis. Subsequently, an aliquot of the suspension of GSSG-r without the ammonium sulphate to which powder GSSG is added is taken. The GSSG-r and GSSG composition is for example maintained under stirring at 37°C. for a period of time comprised between 30 minutes and 3 hours, preferably for 1 hour. Alternatively, it can be conducted at ambient temperature or at a temperature range between ambient temperature and 42°C.

The preparation is thus ready for use. When kept for long periods of time, the preparation is stored at temperatures below -20°C. in aliquots, preferably at -37°C. During use, the aliquot is kept under stirring at a temperature comprised between ambient temperature and 42° C., for example at 37°C. for a period of time comprised between 30 minutes and 3 hours, preferably for 1 hour.

The composition of the experiments is obtained by adding GSSG powder to the suspension of GSSG-r in a variable weight/volume ratio comprised between 1:4 and 1:1, preferably between 1:2.5 and 1:1.5, wherein the suspension of the starting GSSG-r contains a minimum of 100 Enzymatic units/ml.

In particular, the $weight_{GSSG}/UE_{GSSG-r}$/ml ratio, wherein UE_{GSSG-r}/ml are the enzymatic units of Glutathione Reductase per ml of solution, will be comprised between 1:100 and 1:3600, preferably between 1:400 and 1:2000, more preferably between 1:600 and 1:1000 [8].

The components are mixed as described above, so as to obtain the desired GSSG-r/GSSG complex. In a preferred embodiment, said composition comprises 0.500 mg of GSSG and at least 100 Units of GSSG-r per 1 ml.

The expression functional unit is used to describe an aliquot of 4 ml of the composition prepared as described, comprising about 2 mg of GSSG and at least 400 Units of GSSGr.

In the *in vitro* experiments VERO cells are used, that are a cellular line derived from renal epithelial cells of chimpanzees , and they are infected with Poliovirus3, Herpes Simplex 2,Polyomavirus SV40 and Murine Leukaemia Virus (MuLV).

In the *in vivo* experiments two-month old Balb/C mice are used for conducting the experiment. The mice, anaesthetised, are infected-through aerosol-with a swine flu strain adapted to the mouse, A/SW/Finistere/2899/82. The animals are treated with the formulation in question through intranasal method.

Experiments *In vitro*

3.0.1. Determination of the antiviral activity of composition comprising Glutathione Reductase (GSSG-r) and Oxidized Glutathione (GSSG), which we will call GR compound, towards poliovirus 3 in VERO cells.

VERO cells were plated in 48-well micro-plates, in DMEM (Dulbecco's Modified Eagle Medium) culture medium at 10% of FCS (Foetal Cow Serum), 5×10^4 cells/well in 0.5 ml. One plate, which was incubated at 37°C., 5% CO_2 was prepared. The state of growth of the cells in the wells and

Table 1: Anti-virus GR activity. In the horizontal line NC and NC + GR without virus all the cells survived. When Polio virus was added to different dilutions we can note that it was noxious in all the dilutions from -9 to -4 in the three time controls (24h,48h,72h). When GR was added, the viral load was reduced by 4 logarithms, from -9 to -6.

	Dilution (Log10)	24h	48h	72h
NC	NO	0/6	0/6	0/6
Polio	-9	0/3	0/3	3/3
Polio	-8	0/3	3/3	3/3
Polio	-7	0/3	3/3	3/3
Polio	-6	4/4	4/4	4/4
Polio	-5	4/4	4/4	4/4
Polio	-4	3/3	3/3	3/3
NC+GR	NO	0/6	0/6	0/6
Polio + GR	-9	0/3	0/3	0/3
Polio + GR	-8	0/3	0/3	0/3
Polio + GR	-7	0/3	0/3	0/3
Polio + GR	-6	0/3	0/2	0/2
Polio + GR	-5	2/2	2/2	2/2
Polio + GR	-4	3/3	3/3	3/3

Table 2: Herpes simplex 2 GR activity. In the horizontal line NC and NC + GR without virus all the cells survived. When Herpes simplex 2 was added to different dilutions we can note that it was noxious in the dilutions from -2 to 0 in the three time controls (24h,48h,72h).When GR was added, the viral load was reduced by 2 logarithms, from -2 to -1.

	Dilution (Log10)	24h	48h	72h
NC	NO	0/6	0/6	0/6
HSV-2	-5	0/3	0/3	0/3
HSV-2	-4	0/3	0/3	0/3
HSV-2	-3	0/3	0/3	0/3
HSV-2	-2	0/3	2/3	3/3
HSV-2	-1	3/3	3/3	3/3
HSV-2	0	3/3	3/3	3/3
NC + GR	NO	0/6	0/6	0/6
HSV-2 + GR	-5	0/3	0/3	0/3
HSV-2 + GR	-4	0/3	0/3	0/3
HSV-2 + GR	-3	0/3	0/3	0/3
HSV-2 + GR	-2	0/3	0/3	0/3
HSV-2 + GR	-1	0/3	0/3	0/3
HSV-2 + GR	0	3/3	3/3	3/3

Table 3: GR anti Polyomavirus SV40 Activity. In the horizontal line NC and NC + GR without virus all the cells survived. When Polyomavirus SV40 was added to different dilutions we can note that it was noxious in the dilutions from -8 to -4 in the three time controls (14 days, 17 days, 23 days)). When GR was added, the viral load was reduced by 1,5 logarithms from -7 to ½ -8 .

	Dilution (Log10)	14 days	17 days	23 days
NC	NO	0/6	0/6	0/6
SV40	-12	0/2	0/2	0/2
SV40	-11	0/2	0/2	0/2
SV40	-10	0/2	0/2	0/2
SV40	-9	0/2	0/2	0/2
SV40	-8	0/2	1/2	1/2
SV40	-7	2/2	2/2	2/2
SV40	-6	2/2	2/2	2/2
SV40	-4	2/2	2/2	2/2

NC+GR	NO	0/6	0/6	0/6
SV40 + GR	-11	0/2	0/2	0/2
SV40 + GR	-10	0/2	0/2	0/2
SV40 + GR	-9	0/2	0/2	0/2
SV40 + GR	-8	0/2	0/2	0/2
SV40 + GR	-7	0/2	0/2	0/2
SV40 + GR	-6	0/2	2/2	2/2
SV40 + GR	-5	0/2	2/2	2/2
SV40 + GR	-4	0/2	2/2	2/2

Table 4: GR anti retrovirus MuLV Activity. The legend is in the results.

Dilution	**MuLV**		**MuLV + GR 0.67%**			
Log10	Inoculum	Infectious	30 min	Reduction	180 min	Reduction
0	100.000	47.400.000	NT	-	75.000	632
-1	10.000	1.900.000	5.870	323	6.500	292
-2	1.000	475.000	NEG	-	NEG	-
-3	100	23.200	NEG	-	NEG	-
-4	10	4.330	NEG	-	NEG	-
-5	0	NEG	NEG	-	NEG	-

Table 5: Antiviral activity of GR compound.

Virus		**Genome**	**Pericapsid**	**Reduction**
Poliovirus	Polio 3	RNA	NO	4,0 Log 10.000x
Retrovirus	MuLV	RNA	YES	2,7 Log 600x
Poliomavirus	SV40	DNA	NO	1,5 Log 75x
Herpes virus	HSV-2	DNA	YES	2,0 Log 100x

the percentage of confluence, which was of about 90%, was checked after two days. The culture medium was suctioned from all the wells and, as illustrated in the following scheme, 100 µl of the scalar dilutions from 10^{-4} to 10^{-9} of Poliovirus 3-an RNA virus without viral envelope-were added into the wells.

Subsequently the GR compound was added, diluting it to 0.67%, in the wells, as indicated in the diagram below. DMEM culture medium at 2% of FCS was added in all wells to obtain a final volume of 0.5 ml per well. The micro-plate was incubated at 37°C., 5% CO_2. The Poliovirus 3 load was determined after three days of incubation evaluating the cytopathic effect induced by the virus

- **Results**

The load of Polio virus 3 was found to be 1x1010 virus / ml. The GR compound did not show any cytotoxic activity, while it proved an antiviral activity against Poliovirus 3 with a reduction of the viral load by 4 logarithms (Table 1).

3.0.2. Determination of the antiviral activity of GR compound towards the Herpes simplex 2 virus in VERO cells.

VERO cells were plated in 48-well micro-plates, in DMEM culture medium at 10% of FCS, 5×10^4 cells/well in 0.5 ml. One plate, which was incubated at 37° C., 5% CO_2, was prepared. The state of growth of the cells in the wells and the percentage of confluence, which was of about 90%, was checked after two days. The culture medium was suctioned from all plates and, as illustrated in diagram below, 100 µl of the scalar dilutions from 10^0 to 10^{-5} of Herpes simplex 2 virus—an RNA virus without viral envelope—were added into the wells. Subsequently, the GR compound, was added in the wells as indicated in the following diagram (final concentration of 0.67%). DMEM culture medium at 2% of FCS was added in all wells to obtain a final volume of 0.5 ml per well. The micro-plate was incubated at 37°C., 5% CO_2.

- **Results**

The Herpes simplex 2 virus load was determined after three days of incubation evaluating the occurrence of the cytopathic effect induced by the virus. The load was 1×10^3 virus units/ml. The GR compound did not reveal any cytotoxic activity, while it showed an antiviral activity against the Herpes simplex 2 virus with a reduction of the viral load by 2 logarithms (Table 2).

3.0.3. Determination of the antiviral activity of GR compound towards the Polyomavirus SV40 in VERO cells.

The VERO cells were plated in 48-well microplates, in 10% FCS DMEM culture medium: $5x10^4$ cells / well in 0.5 ml. 1 plate was prepared, which was incubated at 37°C with 5% CO_2. After two days the state of growth of the cells in the wells and the percentage of confluence, which was about 90%, were checked. The culture medium was aspirated from all the wells and, as shown in the diagram below, 100 microliters of the scalar dilutions from 10^{-4} to 10^{-11} of the SV40 polyomavirus were inoculated in the wells. Viral adsorption was carried out at room temperature by placing the microplate on a shaker for 2 hours. VERO cells were plated in 48-well micro-plates, in DMEM culture medium at 10% of FCS, 5×10^4 cells/well in 0.5 ml. One plate, which was incubated at 37°C., 5% CO_2 was prepared. The state of growth of the cells in the wells and the percentage of confluence, which was of about 90%, was checked after two days. The culture medium was suctioned from all plates and, as illustrated in the scheme below, 100 µl of the scalar dilutions from 10^{-4} to 10^{-11} of Polyomavirus SV40—a DNA virus without viral envelope—were added into the wells. The viral adsorption was carried out at ambient temperature by positioning the micro-plate on a stirrer for 2 hours. After washing with PBS, the GR compound was added in the wells (final concentration 0,67%), as indicated in the following scheme. DMEM culture medium at 2% of FCS was added in all wells to obtain a final volume of 0.5 ml per well. The micro-plate was incubated at 37°C., 5% CO_2.

- **Results**

The Polyomavirus SV40 load was determined after three weeks of incubation by evaluating the occurrence of transformation foci caused by the virus. The load was $1\times10^{7.5}$ virus units/ml. The GR compound, added three hours after infection (concentration of 0,67%), did not reveal any cytotoxic activity, while it showed an antiviral activity against Polyomavirus SV40 with a reduction of the viral load by 1.5 logarithms. The effect of GR compound was also observed on the occurrence of the viral cytopathic effect, which was delayed by 24 hours on all dilutions (Table 3).

3.0.4.Determination of the antiviral activity of GR compound towards the Murine Leukaemia Virus (MuLV) in Mink Lung cells.

DMEM medium with 2% FCS was added to all wells for a final volume of 0.5 ml per well. The microplate was incubated at 37°C with CO_2. Lung cells were plated in 48-well micro-plates, in DMEM culture medium at 10% of FCS, 5×10^4 cells/ well in 0.5 ml. One plate, which was incubated at 37°C., 5% CO_2 was prepared. The state of growth of the cells in the wells and the percentage of confluence, which was of about 90%, was checked after one day. The culture medium was suctioned from all plates and, as illustrated in the scheme below, 100 µl of the scalar dilutions from 10^{-1} to 10^{-5} of the MuLV—an RNA virus with viral envelope—were added into the wells. Viral adsorption was conducted by incubating the plate at 37°C., 5% CO_2 for 30 minutes. After washing with PBS,the compound GR was added (final concentration of 0.67%) in the wells, immediately or after three hours as indicated in the following scheme. . In particular, the treatment was conducted in a series of wells 30 minutes after the viral infection and, in another series of wells, the same treatment was conducted 180 minutes after the viral infection. DMEM culture medium at 2% of FCS was added in all wells to obtain a final volume of 0.5 ml per well. The micro-plate was incubated at 37°C., 5% CO_2.

- **Results**

The MuLV load was determined after 4 days of incubation collecting the supernatant and conducting the titration of the genomic RNA of MuLV. The load was equivalent to $1\times10^{7.5}$ virus units/ml. The treatment with the GR compound did not reveal any cytotoxic activity (it was added 30 or 180 minutes after the viral infection). GRcompound showed an antiviral activity against MuLV, entirely inhibiting the viral infection when the inoculums were under 10.000. Infections with greater concentrations of MuLV (100.000) revealed an inhibition of the viral replication by 632 times and when inoculums were 10.000 by 323-292 times. In cases where said composition was added 180 minutes instead of 30 minutes after viral infection, it still revealed to be active, though less efficient, with a 292 - fold reduction in the viral titer (Table 4).

3.0.5.**Conclusion:** GR compound concentrations equal to or greater than 25 microliters, corresponding to a 1.14% dilution, added to cell cultures 3 hours after viral infection, cause a visible cytotoxicity in the VERO cells used for viral infection tests. It has been verified that the concentration of non-toxic GR for cell cultures is 0.67%. In this study of evaluation of the antiviral activity of the compound GR, 4 virus prototypes were used: an RNA virus without pericapside (polio-), an RNA virus with pericapside (retro-), a DNA virus without pericapside (polioma-) and a DNA virus with pericapside (Herpes-).

The GR compound demonstrated high antiviral activity against RNA viruses (Table 5) with a maximum reduction in the viral titer for the polio virus (4 Log approximately 10,000x) and the MuLV retrovirus (2.7 Log over 600x). Antiviral activity was around 2 Log (100x) towards the HSV-2 Herpes virus and slightly lower (1.5 Log about 75x) towards the SV40 polyomavirus. A slowdown in viral replication was also observed, suggested by the delayed appearance of the viral cytopathic effect: GR compound delayed the cytopathic effect induced by higher viral loads (>1,000 infectious polioviruses) of about 24 hours, probably due to a possible partial inhibition of the viral replications. This preliminary study confirmed that the GR compound has an important broad-spectrum antiviral activity and therefore stimulates the deepening of the mechanism of action and the optimal conditions of this activity.

Experiment *In vivo*

Final report on the activity carried out to ascertain the antiviral activity of a preparation based on Glutathione Reductase (GSSG-r) and Oxidized Glutathione (GSSG), which we call GR compound in mice, infected with a swine flu virus (H1N1) adapted to mouse.

Experimental protocol: The Balb-c mouse of about two months of life, was selected as an animal model. The animals, within the various tests carried out, were divided into groups. The inoculation of the GR compound was performed intranasally (i.n.) in volumes of 50 microliters/mouse. Vice versa, the infection was carried out both via i.n. (test n ° 1) and for aerosol (test n ° 2). The latter route was the most valid way in conferring the infection on the majority of animals [9].

Virus: A strain of swine flu virus was used: A / SW / Finistere / 2899/82 which was adapted to the mouse. This adaptation was carried out by repeatedly inoculating groups of 10 subjects by i.n. The animals were kept under observation and, in the absence of mortality, the same were sacrificed. Lung tissue, the target organ of the virus,

Table 1: Outcomes of Mouse treated with GR and infected with the swine flu virus endonasally.

Group	Mouse	Exctracted organ weight (mg)	Quantity (n° copies)	Quantity (copies/mg tissue)	Outcome
Group 1 50 microliters of GR	Mouse 1	55,5	3,63E + 07	6,53E + 05	+
	Mouse 2	71,6	2,13E + 02	2,97E + 00	-
	Mouse 3	83,9	4,08E + 02	4,87E + 00	-
	Mouse 4	71	2,40E + 06	3,38E + 04	+
	Mouse 5	95,3	1,02E + 04	1,07E + 02	-/+
	Mouse 6	93,8	6,95E + 02	7,41E + 00	-
	Mouse 7	70,7	5,44E + 08	7,69E + 06	++
Group 2 25 microliters of GR	Mouse 1	37,6	7,65E + 01	2,04E + 00	-
	Mouse 2	63,3	1,26 + E02	1,99E + 00	-
	Mouse 3	83,9	0,00E + 00		-
	Mouse 4	78,6	2,70E + 06	3,43E + 04	+
	Mouse 5	89,6	1,60E + 06	1,78E + 04	+
	Mouse 6	98,5	7,06E + 06	7,17E + 04	+
	Mouse 7	77,2	6,08E + 03	7,88E + 01	-
Group 3 12,5 microliters of GR	Mouse 1	81,6	1,57E + 06	1,92E + 04	+
	Mouse 2	58,2	2,72E + 01	4,68E - 01	-
	Mouse 3	81,4	1,33E + 06	1,63E + 04	+
	Mouse 4	87,7	0,00E + 00		
	Mouse 5	81,4	1,14E + 03	1,40E + 01	-
	Mouse 6	97	7,83E + 05	8,07E + 03	+/-
	Mouse 7	143,5	1,46E + 02	1,02E + 00	-
Group 4 Only infected	Mouse 1	91,6	2,36E + 02	2,58E + 00	-
	Mouse 2	9,2	6,83E + 00	7,42E - 01	-
	Mouse 3	64,7	0,00E + 00		-
	Mouse 4	102,4	3,29E + 03	3,21E + 01	-
	Mouse 5	41,2	0,00E + 00		-
	Mouse 6	93	0,00E + 00		-
	Mouse 7	84	0,00E + 00		-
Group 5 50 microliters of phys. sol.	Mouse 1	76,6	2,41E + 01	3,14E-01	-
	Mouse 2	83,6	7,46E + 06	8,92E+04	+
	Mouse 3	91,8	5,31E + 01	5,79E-01	-
Group 6 25 microliters of phys. sol.	Mouse 1	97	8,89E + 01	9,17E-01	-
	Mouse 2	97,6	4,95E + 06	5,07E+04	+
	Mouse 3	90,6	2,44E + 04	2,70E + 02	-/+
Group 7 12,5 microliters of phys. sol.	Mouse 1	9,6	0,00E + 00		-
	Mouse 2	86,7	1,19E + 02	1,37E + 00	-
	Mouse 3	92,6	9,54E + 02	1,03E + 01	-

The test was then repeated by infecting the animals by aerosol rather than by intranasal inoculation.

Table 2: Outcomes of mice treated with GR and infected with the swine flu virus by aerosol.

Group n°	Mice n°	Treatment	Biological outcome: No. of deaths / No. of mice in the group	Molecular biology outcome cycle threshold range
1	10	Infection and treatment with the product concerned (50 microliters)	3/10	14,35-16,70
2	10	Infection and treatment with the product concerned (25 microliters)	9/10	13,00-19,88; 1 negative mice
3	10	Infection	8/10	15,45-19,11; 1 animal with value 27.09 probably has contracted a light infection
4	5	Infection and treatment with phys. sol. (50 microliters)	5/5	16,02-18,65
5	5	Infection and treatment with phys. sol. (25 microliters)	4/5	15,10-17,50; 1 animale with value 30,10 probably has contracted a light infection

The results of this last test would indicate how GR compound at the concentration of 50 microliters/mouse was able to significantly reduce lethality compared to the other groups treated with a lower concentration.

was collected and the viral presence ascertained by real-time PCR reaction. The infection was carried out using the lung tissue pool of sacrificed animals which were positive in the molecular biology test. The samples were subjected to homogenization, centrifugation, collection of the supernatant and quantification of the viral nucleic acid by Real time PCR.

Operational scheme

Experiment n ° 1: Twenty-one mice divided into 3 groups treated with GR compound 3 times a day (4-hour intervals) / 4 days (n ° 7 mice / group) inoculated via i.n. and choosing different concentrations. A fourth group representing the control and consisting of 7 subjects, was only subjected to the experimental infection. 3 other groups (each consisting of 3 mice) were treated with physiological solution and subjected to infection.

Virus: administered via i.n. (50 microliters / mouse)

Group 1: infected and treated with GR (50 microliters)

Group 2: infected and treated with GR (25 microliters)

Group 3: infected and treated with GR (12.5 microliters)

Group 4: Control only infected (7 mice)

3 groups treated 3 times a day with physiological solution (4 hour intervals) / 4 days (n ° 3 mice / group):

Group 5: infected and treated with physiological solution (50 microliters)

Group 6: infected and treated with physiological solution (25 microliters)

Group 7: infected and treated with physiological solution (12.5 microliters)

Sacrifice after 4-5 days. Real time PCR performed on the lung tissue of all animals.

Results: The results of the test carried out, indicated in the table below, are not to be considered reliable because the controls were not infected and therefore the results are not comparable.

Experiment n ° 2: Performed on 40 Balb-c mice. Virus: swine flu H1N1 adapted to the mouse: 50 microliters / mouse; administered by aersol in anesthetized animals.

2 groups treated with the product concerned 3 times a day (4 hour intervals) / 4 days

Group 1: infected and treated with GR (50 microliters) (10 mice)

Group 2: infected and treated with GR (25 microliters) (10 mice)

Group 3: infected control only (10 mice)

2 groups treated 3 times a day with physiological solution (4 hour intervals) / 4 days (n ° 5 mice / group):

Group 4: infected and treated with physiological solution (50 microliters) (5 mice)

Group 5: infected and treated with physiological solution (25 microliters) (5 mice)

Sacrifice 4-5 days after the last treatment and corresponding to 8-9 days after infection.

Real time PCR performed on the lung tissue of all animals.

Results: The animals came to death in the first 4-5 days of infection; consequently, the outcome of molecular biology, in this context, is not significant.

4. The effectiveness of the product must therefore be assessed on the difference in lethality (Table 2).

	1	2	3	4	5	6	7	8
A	NC	NC + GR	10^{-9} + GR	10^{-9} + GR	10^{-9} + GR	10^{-9}	10^{-9}	10^{-9}
B	NC	NC + GR	10^{-8} + GR	10^{-8} + GR	10^{-8} + GR	10^{-8}	10^{-8}	10^{-8}
C	NC	NC + GR	10^{-7} + GR	10^{-7} + GR	10^{-7} + GR	10^{-7}	10^{-7}	10^{-7}
D	NC	NC + GR	10^{-6} + GR	10^{-6} + GR	10^{-6} + GR	10^{-6}	10^{-6}	10^{-6}
E	NC	NC + GR	10^{-5} + GR	10^{-5} + GR	10^{-5} + GR	10^{-5}	10^{-5}	10^{-5}
F	NC	NC + GR	10^{-4} + GR	10^{-4} + GR	10^{-4} + GR	10^{-4}	10^{-4}	10^{-4}

Column 1) NC = Negative Control; 2) NC + GR: The compound GR diluted 0.67% was added to show that there is no toxicity in that dilution; 3-5) In these experimental lines, dilutions of Polio virus from 10^{-4} to 10^{-9} + the compound GR were added; 6-8) In these lines, only dilutions of Polio virus from 10^{-4} to 10^{-9} were added .

	1	2	3	4	5	6	7	8
A	NC	NC + GR	10^{-5} + GR	10^{-5} + GR	10^{-5} + GR	10^{-5}	10^{-5}	10^{-5}
B	NC	NC + GR	10^{-4} + GR	10^{-4} + GR	10^{-4} + GR	10^{-4}	10^{-4}	10^{-4}
C	NC	NC + GR	10^{-3} + GR	10^{-3} + GR	10^{-3} + GR	10^{-3}	10^{-3}	10^{-3}
D	NC	NC + GR	10^{-2} + GR	10^{-2} + GR	10^{-2} + GR	10^{-2}	10^{-2}	10^{-2}
E	NC	NC + GR	10^{-1} + GR	10^{-1} + GR	10^{-1} + GR	10^{-1}	10^{-1}	10^{-1}
F	NC	NC + GR	10^{0} + GR	10^{0} + GR	10^{0} + GR	10^{0}	10^{0}	10^{0}

Column 1) NC = Negative control; 2) NC + GR: The compound GR diluted 0.67% was added to show that there is no toxicity in that dilution; 3-5) In these experimental lines dilutions of Herpes simplex 2 from 10^{0} to 10^{-5} + the compound GR were added; 6-8) In these lines only dilutions of Herpes simplex 2 from 10^{0} to 10^{-5} were added.

	1	2	3	4	5	6	7	8
A	NC	NC + GR	10^{-11} + GR	10^{-11} + GR	10^{-5} + GR	10^{-5} + GR	10^{-12}	10^{-12}
B	NC	NC + GR	10^{-10} + GR	10^{-10} + GR	10^{-4} + GR	10^{-4} + GR	10^{-11}	10^{-11}
C	NC	NC + GR	10^{-9} + GR	10^{-9} + GR	NC	NC	10^{-10}	10^{-10}
D	NC	NC + GR	10^{-8} + GR	10^{-8} + GR	NC	NC	10^{-9}	10^{-9}
E	NC	NC + GR	10^{-7} + GR	10^{-7} + GR	10^{-4}	10^{-4}	10^{-8}	10^{-8}
F	NC	NC + GR	10^{-6} + GR	10^{-6} + GR	10^{-6}	10^{-6}	10^{-7}	10^{-7}

Column 1) NC = Negative control; 2) NC + GR: the compound GR diluted 0.67 % was added to show that there is no toxicity to that dilution; 3,4) In these experimental lines dilutions of Polyoma virus SV40 from 10^{-6} to 10^{-11} + the compound GR were added; 5,6) These lines are mixed : A,B) Dilutions of Plyomavirus SV40 from 10^{-4} to 10^{-5} + GR were added; C,D) Only NC; E,F) Only dilutions of Polyomavirus from 10^{-4} to 10^{-6} were added; 7,8) Dilutions of Polyomavirus SV40 from 10^{-7} to 10^{-12} were added.

	1	2	3	4	5	6	7	8
A	NC	NC + GR	10^{-5} + GR	10^{-5} + GR	10^{-5} + GR	10^{-5}	10^{-5}	10^{-5}
B	NC	NC + GR	10^{-4} + GR	10^{-4} + GR	10^{-4} + GR	10^{-4}	10^{-4}	10^{-4}
C	NC	NC + GR	10^{-3} + GR	10^{-3} + GR	10^{-3} + GR	10^{-3}	10^{-3}	10^{-3}
D	NC	NC + GR	10^{-2} + GR	10^{-2} + GR	10^{-2} + GR	10^{-2}	10^{-2}	10^{-2}
E	NC	NC + GR	10^{-1} + GR	10^{-1} + GR	10^{-1} + GR	10^{-1}	10^{-1}	10^{-1}
F	NC	NC + GR	NC	NC	NC	NC	NC	NC

Column 1) NC = Negative control; 2) NC + GR: compound GR diluted 0.67 % was added to show that there is no toxicity to that dilution; 3-5) In these experimental lines dilutions of MuLV from 10^{-1} to 10^{-5} + the compound GR were added, except in the F line where only NC was added; 6-8) In these lines dilution of MuLV from 10^{-1} to 10^{-5}, except in the F line.

RESULTS

This technology is a way to dominate virus.

CONCLUSIONS

An entirely new and surprising approach is that claimed in the present invention of this GR compound (GSSG-r + GSSG), that is active and safe in the treatment of viral diseases and , more generally, in the reduction of cellular damage caused by the loss of the oxide-reductive balance of the cell.

The remarkable wide spectrum antiviral properties of this compound, suggest trying experiments against HIV but expecially towards Covid 19 that is exhausting the world in this moment, because its efficacy could be very probable.

In the future, the use of the compound is desirable against radiation and the toxicity of the radicals produced by the radiolysis of cellular water, or in other pathologic

condition where there is a misbalanced GSH:GSSG ratio, such as tumours, cystic fibrosis, neurodegenerative diseases.

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