PARVOVIRUS FORMULATION FOR TREATING TUMORS

The present invention relates to a parvovirus formulation where the parvovirus is present in a concentration of at least 1 x 10^9 pfu/ml in a pharmaceutically acceptable carrier containing 40-50% Iodixanol (w/v), 0.7-0.9 mmol $CaCl_2$ x 2 H_2O, 50-60 mmol NaCl, 0.9-1.2 mmol KCl, 0.7-0.95 mg/ml Tromethamine and 0.05-0.15 mg/ml Edetate calcium disodium. The carrier has a viscosity between 3 to 5 mPa·s at 37°C to 40°C.

Cancer is the second leading cause of death worldwide. It has been estimated that half of men and one third of women will be diagnosed with some form of cancer during their lifespan. Moreover, because cancer is predominantly a disease of aging, the number of cancer deaths worldwide is predicted to increase about 45% from 2007 to 2030 (from 7.9 million to 11.5 million deaths) due to the increase proportion of elderly people (WHO estimates, 2008). Cancer is also the most costly disease. The latest estimates from the National Cancer Institute showed that the overall economic cost of cancer in the U.S. in 2007 was $226.8 billion and unless more successful preventive interventions, early detection and more efficient treatments will be developed, this already huge economic burden is expected to further grow during the next two decades. Despite significant progresses in the prevention, detection, diagnosis and treatment of many forms of cancer, which is testified by an increase of the percentage of 5-years cancer survivals in U.S. and in Europe over the last thirty years, some tumour types, such as pancreatic, liver, lung, brain remain orphan of effective treatments calling for the development of new therapeutic options. Oncolytic viruses, which exploit cancer-specific vulnerabilities to kill cancer cells

while sparing normal cells are fast emerging as promising tools for fighting cancer. No less than twelve different oncolytic viruses are currently undergoing phase I-III clinical trials against various malignancies used alone or in combination with other anticancer agents. Among them, the oncolytic rat parvovirus H-1PV is currently evaluated for safety and first signs of efficacy in a phase I/IIa clinical trial in patients having recurrent glioblastoma multiforme (GBM) (Geletneky et al, BMC Cancer 2012, pp. 99).

H-1PV is a small (~25 nm in diameter), non-enveloped icosahedral particle containing a 5.1 kb long single-stranded DNA genome. The genomic organization of H-1PV consists of two transcriptional units under the control of two promoters, the P4 early promoter and P38 late promoter. P4 regulates the expression of the gene encoding the non-structural (NS) proteins (NS1 and NS2) and the P38 the one encoding the capsid (VP) proteins (VP1, VP2, VP3). The virus multiplies preferentially in fast dividing cancer cells. This onco-selectivity is not based on a better uptake of the virus by cancerous cells, but rather is due to the fact that cancer cells overexpress factors, such as cyclin A, E2F, or CREB/ATF, required for virus DNA replication. Cancer therapy using a parvovirus and its combination with chemotherapy or an HDAC inhibitor has been recently described (WO 2009/083232 A1; WO 2011/113600 A1).

One frequent problem of local therapy of tumors by injection or infusion of oncolytic viruses is the targeted distribution of the inoculum and a loss of active substance. In particular, when a catheter is used the distribution takes place along the catheter track due to an area of diminished resistance along the path of the catheter through the tissue. This phenomenon is also known as backflow.

Therefore, it is the object of the present invention to provide a pharmaceutical composition which does not suffer under the problem of backflow upon administration and is stable during a storage time of at least 12 months, even under elevated temperature conditions for several days and UV treatment.

According to the invention this is achieved by the subject matters defined in the claims.

Thus, the present invention provides a pharmaceutical composition comprising (a) a parvovirus H1 (H-1PV) or a related rodent parvovirus selected from the group consisting of LuIII, Mouse minute virus (MMV), Mouse parvovirus (MPV), Rat minute virus (RMV), Rat parvovirus or Rat virus (RV) in a concentration of at least 1 x 10^9 pfu/ml and (b) a pharmaceutically acceptable carrier containing 40-50% Iodixanol (w/v), 0.7-0.9 mmol $CaCl_2$ x 2 H_2O, 50-60 mmol NaCl, 0.9-1.2 mmol KCl, 0.7-0.95 mg/ml Tromethamine and 0.05-0.15 mg/ml Edetate calcium disodium. The pharmaceutical acceptable carrier has a viscosity between 3 and 5mPa·s at 37 to 40°C.

Brief description of the drawings

Figure 1: H-1PV in 48% Visipaque/Ringer solution demonstrates the targeted distribution of the inoculum around the tip of the catheter without signs of random diffusion

Figure 2: H-1PV is stable for more than 24 months in 48% Visipaque/ringer formulation

Figure 3: Electron micrographs showing insular H-1PV particles in 48% visipaque/ringer formulation

a) H-1PV charge 1, **b)** H-1PV charge 2

Figure 4: Clinical Trial Design

Figure 5: Virus Concentration in Iodixanol/Ringer solution [73.62 % Visipaque™ 320(GE Healthcare) mixed with 26.38% Ringer solution]

Figure 6: Treatment of H-1 PV in Iodixanol/Ringer solution at elevated temperature

Figure 7: UV-Treatment of H-1 PV in two Iodixanol/Ringer solutions (A,B) and Tris-EDTA buffer (C)

Thus, the present invention provides a pharmaceutical composition comprising (a) a parvovirus H1 (H-1PV) or a related rodent parvovirus selected from the group consisting of LuIII, Mouse minute virus (MMV), Mouse parvovirus (MPV), Rat minute virus (RMV), Rat parvovirus or Rat virus (RV) in a concentration of at least 1 x 10^9 pfu/ml and (b) a pharmaceutically acceptable carrier having a viscosity between 3 and 5 mPa·s at 37 to 40°C and containing 40-50% Iodixanol (w/v), 0.7-0.9 mmol $CaCl_2 \times 2\ H_2O$, 50-60 mmol NaCl, 0.9-1.2 mmol KCl, 0.7-0.95 mg/ml Tromethamine and 0.05-0.15 mg/ml Edetate calcium disodium.

The term "parvovirus" as used herein comprises wild-type or modified replication-competent derivatives thereof. Suitable modified parvoviruses which can be used for actively producing said parvoviruses and which are useful for therapy, are readily determinable within the skill of the art based on the disclosure herein, without undue empirical effort.

According to the present invention, the parvovirus of the composition includes parvovirus H1 (H1PV) or a related parvovirus

such as LuIII, Mouse minute virus (MMV), Mouse parvovirus (MPV), Rat minute virus (RMV), Rat parvovirus (RPV) or Rat virus (RV).

According to the invention the parvovirus is present in an effective dose in a concentration of or higher than 1×10^9 pfu/ml and combined with a pharmaceutically acceptable carrier containing 40-50% Iodixanol (w/v), 0.7-0.9 mmol $CaCl_2 \times 2\ H_2O$, 50-60 mmol NaCl, 0.9-1.2 mmol KCl, 0.7-0.95 mg/ml Tromethamine and 0.05-0.15 mg/ml Edetate calcium disodium. In particular, the carrier contains 45-49% Iodixanol (w/v), 0.75-0.85 mmol $CaCl_2 \times 2\ H_2O$, 50-55 mmol NaCl, 0.95-1.1 mmol KCl, 0.8-0.9 mg/ml Tromethamine and 0.05-0.10 mg/ml Edetate calcium disodium. In a most preferred embodiment the carrier contains 48% Iodixanol (w/v), 0.81 mmol $CaCl_2 \times 2\ H_2O$, 52.80 mmol NaCl, 1.06 mmol KCl, 0.88 mg/ml Tromethamine and 0.07 mg/ml Edetate calcium disodium.

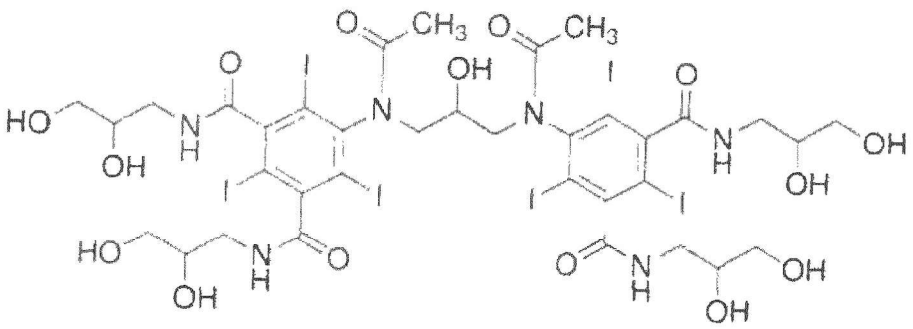
"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredients and that is not toxic to the patient to whom it is administered. In addition, to the above defined carrier composition, the pharmaceutical formulation according to the present invention may contain additional pharmaceutical carriers. Examples of suitable additional pharmaceutical carriers are well known in the art and include mannitol, sorbitol, glucose or sucrose. Such carriers can be formulated by conventional methods.

An "effective dose" refers to amounts of the active ingredients that are sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. An "effective dose" useful for treating and/or preventing these diseases or disorders may be determined using methods known to one skilled in the art. In addition, to ensure storage stability of the formulation, the active ingredient, i.e. the parvovirus,

should be present in a concentration of at least 1 x 10^9 pfu/ml, particularly at least 5 x 10^9 pfu/ml, 1 x 10^{10} pfu/ml or 1x 10^{11} pfu/ml. In a preferred embodiment the concentration is between 1x 10^9 pfu/ml and 1 x 10^{10} pfu/ml, in particular 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x or 9x 10^9 pfu/ml. In another preferred embodiment the the concentration is between 1x 10^{10} pfu/ml and 1 x 10^{11} pfu/ml, in particular 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x or 9x 10^{10} pfu/ml.

According to the present invention, the viscosity of the carrier is between 3.5 to 4.5 mPa·s at 37 to 40°C. More preferred is a carrier wherein the viscosity of the carrier is between 4 and 5 mPa·s. Even more preferred is a carrier having a viscosity of about 4.5 mPa·s. This viscosity resembles the viscosity of blood (Rosenson et al., Clinical Chemistry, 42:8, pp. 1189-1195 (1996); http: www.ucke.de/christian/physik/medprak/Viskositaet.pdf: page 84, Table 2).

According to a preferred embodiment of the present invention, the carrier is iodixanol in Ringer solution that is prepared by mixing , 73,62% VISIPAQUE™ 320 (GE Healthcare) with 26,38% Ringer solution. VISIPAQUE™ 320 (GE Healthcare) contains 652 mg/ml Iodixanol (= 65,2% iodixanol) so that the iodixanol concentration after mixing with Ringer solution is 48%. "Iodixanol" is a synonym for "Visipaque" (for human injection use) or "Iodixanolum" (research grade). The chemical structure is



The IUPAC name is 5-[acetyl-[3-[N-acetyl-3,5-bis(2,3-dihydroxypropylcarbamoyl)2,4,6,-triiodoanilino]2-hydroxypropyl]amino]-1-N,3,N-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide. The CAS number is 92339-11-2. It is also a well known contrast agent for CT imaging.

Administration may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, intradermal or intratumoral administration. The route of administration, of course, depends on the kind of therapy and the kind of compounds contained in the pharmaceutical composition. A preferred route of administration is intratumoral or intravenous administration. The dosage regimen is readily determinable within the skill of the art, by the attending physician based on patient data, observations and other clinical factors, including for example the patient's size, body surface area, age, sex, the particular parvovirus, cell etc. to be administered, the time and route of administration, the tumor type and characteristics, general health of the patient, and other drug therapies to which the patient is being subjected.

The pharmaceutical composition of the present invention is useful for the treatment of any tumor type, in particular (but not exclusively) brain tumor (e.g. glioma and glioblastoma), pancreatic carcinoma, cervical carcinoma, lung cancer, head and neck cancer, breast cancer or colon cancer.

Patients treatable by the pharmaceutical composition according to the invention include humans as well as non-human animals. Examples of the latter include, without limitation, animals such as cows, sheep, pigs, horses, dogs, and cats.

From the clinical data as shown in Example 1 it can be concluded that the use of Visipaque 48% allows for (i) precise visualization of the inoculum by CT, (ii) precise local delivery as a result of the higher viscosity and (iii) the absence of backflow even when manual injection and no prolonged and automated injection such as convection enhanced delivery (CED) was used. This favorable pattern of distribution is most likely due to the different physical properties of Visipaque 48% compared with watery solutions. Vispaque 48% in Ringer solution with a refraction index of 1,41 has a viscosity at 37°C of 4 mPa·s, water at 37°C of ~0,7 mPa·s. The human blood at 37°C has a viscosity of 3-5 mPa·s. Therefore, the injection formulation is close to blood that means in a physiological value of blood.

The inventors have found out that the virus concentration has an influence on the stability of the formulation (Fig. 5). To ensure a storage stability of at least 12 months the virus concentration should be at least 1 x 10^9 pfu/ml in a 48% Iodixanol/Ringer solution that has been obtained by mixing 73,62% (w/v)VISIPAQUE™ 320 (GE Healthcare) with 26,30% (w/v) Ringer solution. The obtained solution contains 48% Iodixanol (w/v), 0.81 mmol $CaCl_2$ x 2 H_2O, 52.80 mmol NaCl, 1.06 mmol KCl, 0.88 mg/ml Tromethamine and 0.07 mg/ml Edetate calcium disodium.

In a preferred embodiment the storage stability is at least 18 months, at least 24 months, at least 30 months, at least 36 months or even up to 48 months.

As can be concluded from Figs. 6 and 7, the virus in 48% Iodixanol/Ringer solution remains active several days even after an temperature increase and an UV radiation.

The below examples explain the invention in more detail.

Example 1

Visipaque 48% avoids backflow

A clinical phase I/IIa trial on 18 patients suffering from recurrent malignant gliomas was initiated. This trial aims to investigate the safety, biodistribution, maximum tolerated dose and signs of anti-tumor activity of parvovirus H-1. According to preclinical data the parvovirus will not only include intratumoral virus application but also intravenous treatment.

The application of parvovirus H-1 (GMP-grade preparation) in 48% Visipaque was performed in 2 groups of 12 (group I) and 6 patients (group II). The route of administration differs between group 1 and group 2 (Figure 4).

Within each group the mode of application is identical, but the dose will be increased if no dose limiting events are observed. In group I the parvovirus H-1 (also called "investigational medicinal product"; IMP) was administered in four dose levels and in group II in 2 dose levels (Table 1).

Table 1 Dose schedule for both study groups

GROUP I Escalation Level	Study Time	Dose and route of administration	Duration
Level 1 Total dose: 1×10^6 pfu	Day 1	5×10^5 pfu, intratumoral (via catheter)	15 minutes
	Day 10	5×10^5 pfu, intracerebal (direct injection at multiple locations of resection wall)	15-30 minutes
Level 2 Total dose: 5×10^7 pfu	Day 1	2.5×10^7 pfu, intratumoral (via catheter)	15 minutes
	Day 10	2.5×10^7 pfu, intracerebal (direct injection at multiple locations of resection wall)	15-30 minutes
Level 3 Total dose: 1×10^9 pfu	Day 1	5×10^8 pfu, intratumoral (via catheter)	15 minutes
	Day 10	5×10^8 pfu, intracerebral (direct injection at multiple locations of resection wall)	15-30 minutes
Level 4 Total dose: 5×10^9 pfu	Day 1	2.5×10^9 pfu, intratumoral (via catheter)	15 minutes
	Day 10	2.5×10^9 pfu, intracerebal (direct injection at multiple locations of resection wall)	15-30 minutes
GROUP II Escalation Level			

Level 2 Total dose: 5×10^7	Day 1 – 5	0.5×10^7 pfu, intravenous infusion	2 hours
	Day 10	2.5×10^7 pfu, intracerebral (direct injection at multiple locations of resection wall)	15-30 minutes
Level 3 Total dose: 1×10^9 pfu	Day 1 – 5	1×10^8 pfu, intravenous infusion	2 hours
	Day 10	5×10^8 pfu, intracerebral (direct injection at multiple locations of resection wall)	15-30 minutes

In group 1 the patients received the IMP on day 1 via image guided injection into the tumor tissue. On this day the patient is injected with 50% of the intended overall dose. After an observation period of 9 days the tumor was resected on day 10. After tumor removal the second half of the dose was administered into the walls of the resection cavity by direct injection. With this injection during open surgery the administration of the IMP is completed and no additional virus application was performed.

In group 2 the initial administration of the IMP was via the intravenous route. Subjects received 50% of the intended dose by 5 infusions on days 1 to 5, each infusion containing 10% of the total dose. After the last infusion on day 5 there is an observation period until day 9 and on day 10 tumor resection was performed as in group 1. In analogy to group 1, patients receive the second half of the dose by injection in the tissue surrounding the tumor cavity after tumor removal and no further virus injections were performed in each individual during the course of the trial.

After tumor resection virus was reapplied in the walls of the tumor cavity.

As shown in Figure 1 the use of Visipaque 48% (VISIPAQUE™ 320, GE Healthcare, Germany) and Ringer solution (IDT Biologika GmbH, Germany) as carrier solution did not result in any significant backflow in all 12 patients. The CT was performed within 30 minutes after completion of injection. The volume of injection

was between 0,5 ml to 1,2 ml per inserted catheter (3 patients were treated with 2 catheters). The injection was performed manually over a period of 30 min per catheter and standard catheters for cyst puncturing with one opening at the end of the catheter were used in all cases. Figure 1 also demonstrates the targeted distribution of the inoculum around the tip of the catheter without signs of random diffusion.

From this clinical data it can be concluded that the use of Visipaque 48% allows for (i) precise visualization of the inoculum by CT, (ii) precise local delivery as a result of the higher viscosity and (iii) the absence of backflow even when manual injection and no prolonged and automated injection such as convection enhanced delivery (CED) was used. This favorable pattern of distribution is most likely due to the different physical properties of Visipaque 48% compared with watery solutions. Vispaque 48% in Ringer solution with a refraction index of 1,41 has a viscosity at 37°C of 4 mPa·s, water at 37°C of ~0,7 mPa·s. The human blood at 37°C has a viscosity of ~4,5 mPa·s. Therefore the injection formulation is close to blood, that means in a physiological value of blood.

Example 2

Stability

The H-1PV virus (stored by -20°C or ≤-60°C in 48% Iodixanol/Ringer solution obtained by mixing 73.62% (w/v) VISIPAQUE™ 320 (GE Healthcare) with 26.30% (w/v) Ringer solution (Delta Select GmbH, Dreieich, Germany)) is stable in activity measured with plaque forming units (PFU) for more than 2 years. Plaque assays were done essentially as described by Tattersall

and Bratton (J. Virol. 46 (1983), 944-55). NB-324K cells were grown in monolayer cultures in MEM medium containing 5% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. They were infected at 60% confluence with serial dilutions of H-1PV and incubated for 1 h at 37°C. Then the inoculum was replaced with a bacto-agar overlay (1.7% in MEM containing 5% FBS). On day four post-infection, living cells were stained for 18-24 h by addition of 0.02% toluylene red staining solution (Sigma, Germany) containing bacto-agar (Becton Dickinson, Germany). The dishes were incubated at 37°C under 5% CO_2. Plaque-forming units were counted 5 days post-infection on a light box and their concentration expressed in PFU/ml.

The results are shown in Fig. 5. There it can be seen that a virus concentration less than 1 x 10^8 pfu/ml is more instable in 48% Iodixanol/Ringer solution than a higher virus concentration of about 1 x 10^9 or 1 x 10^{10} pfu/ml.

Example 3

Insular H-1PV particles: The electron microscopy pictures show that the H-1PV virus is not aggregated in the formulation according to the invention

For qualitative analysis of virus preparations, electron microscopy pictures were taken. For this, 5 µl virus suspension was added to a ready-to-use carbon-coated copper grid and incubated for 2 min. The grid was then washed with 5 µl bidest water and coated with 2% uranyl acetate for 30 s. The drops were absorbed from the grid with Whatman 50 filter paper and the grid was dried for approximately 1 min. Photos were taken with a Zeiss transmission electron microscope at 20,100 x magnification.

Example 4

Influence of temperature increase to H-1PV in 48% Visipaque/Ringer solution

Parameter:

Volume: 100µl

Vial: 500 µl screw cap

Temperatur: 37°C, 50°C

Incubation time: 1h, 4h

Further storage: 4°C

Concentration H-1PV: 2,3 x 10^{10} PFU/ml:

		Time 1	Time 2
Temp.	37°C	60 min	240 min
	50°C	60 min	240 min

The results are shown in Fig. 6.

Fig. 6 shows that the virus is still alive after 1-4 hours at 37°C and 50°C treatment. Even after 4 weeks in 4°C the H-1 PV is stable.

Example 5

Influence of UV radiation to H-1PV in 48% Visipaque/Ringer solution

UV-Deactivation of H-1PV in Visipaque (48% Iodixanol)/Ringer solution and Visipaque (24% Iodixanol)/Ringer solution

exp# 1 (UV 254 nm, 0.35mW/cm²)

		PFU/ml	Vg/ml	PP/ml
Charge 1 in Visipaque(48% Iod)/Ringer	before UV	1.80E+09	1.40E+12	1.00E+13
volume:500µl	after UV	2.00E+09	1.30E+12	8.00E+12
Charge 2 in Visipaque (48% Iod)/Ringer	before UV	1.30E+08	7.30E+10	2.00E+11
volume:500 µl	after UV	6.40E+07	8.10E+10	2.10E+11

exp# 2 (UV 254 nm, 0.35mW/cm²)

		PFU/ml	Vg/ml	PP/ml
Charge 1 in Visipaque(48% Iod)/Ringer	before UV	1.80E+09	1.40E+12	1.00E+13
volume: 250µl	after UV	>3E7	1.40E+12	1.00E+13
Charge 2 in Visipaque (48% Iod)/Ringer	before UV	1.30E+08	7.30E+10	2.00E+11
volume: 250µl	after UV	>3E7	7.30E+10	3.10E+11
Charge 3 in VTE	before UV	3.00E+07	5.40E+11	4.10E+13
	after UV	<1E2	2.80E+10	5.60E+13

exp# 3 (UV 254 nm, 0.35mW/cm²)

		PFU/ml	Vg/ml	PP/ml
Charge 1 in Visipaque(48% Iod)/Ringer	before UV	1.40E+09	9.50E+11	1.00E+13
volume: 100µl	after UV	2.40E+09	1.00E+12	1.10E+13
Charge 2 in Visipaque (48% Iod)/Ringer	before UV	5.10E+07	5.00E+10	2.00E+11
volume: 100µl	after UV	5.80E+07	5.00E+10	2.00E+11

exp# 4 (UV 254 nm, 2.4mW/cm²)

		PFU/ml	Vg/ml	PP/ml
Charge 1 in Visipaque(48% Iod)/Ringer	before UV	1.70E+09	1.40E+12	9.80E+12
volume: 100µl	after UV	1.80E+09	1.70E+12	7.70E+12
Charge 2 in Visipaque(24% Iod)/Ringer	before UV	4.60E+09	6.80E+11	1.00E+13
volume: 100µl	after UV	1.20E+09	4.10E+11	3.50E+11

The results are shown in Fig. 7. The H-1 PV virus in Visipaque (48% Iodixanol)/Ringer solution is protected against UV treatment through the formulation. The stability of the virus in Visipaque (24% Iodixanol)/Ringer solution shows a half log decrease after UV treatment. In an aqueous formulation (Tris-EDTA buffer = VTE) the virus could be deactivated.

Claims

1. Pharmaceutical composition comprising (a) at least 2 x 10^9 pfu/ml of parvovirus H1 (H-1PV) or a related rodent parvovirus selected from the group consisting of LuIII, Mouse minute virus (MMV), Mouse parvovirus (MPV), Rat minute virus (RMV), Rat parvovirus or Rat virus (RV) and (b) a pharmaceutically acceptable carrier containing 40-50% Iodixanol (w/v), 0.7-0.9 mmol $CaCl_2$ x 2 H_2O, 50-60 mmol NaCl, 0.9-1.2 mmol KCl, 0.7-0.95 mg/ml Tromethamine and 0.05-0.15 mg/ml Edetate calcium disodium.

2. The pharmaceutical composition of claim 1, wherein the pharmaceutical acceptable carrier contains 48% Iodixanol (w/v), 0.81 mmol $CaCl_2$ x 2 H_2O, 52.80 mmol NaCl, 1.06 mmol KCl, 0.88 mg/ml Tromethamine and 0.07 mg/ml Edetate calcium disodium.

3. The pharmaceutical composition of claim 1 or 2, wherein the parvovirus concentration is between 2 x 10^9 pfu/ml and 1 x 10^{10} pfu/ml.

4. The pharmaceutical composition of any one of claims 1 to 3 for use in treating a tumor by intratumoral or intravenous injection.

5. The pharmaceutical composition of any one of claims 1 to 3 for the use according to claim 4, wherein the tumor is a brain tumor, pancreatic carcinoma, cervical carcinoma, lung cancer, head and neck cancer, breast cancer or colon cancer.