PART 1 (COUNCIL DECISION 2002/813/EC)

SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

In order to tick one or several possibilities, please use crosses (meaning x or X) into the space provided as (.)

A. General information

1. Details of notification

(a) Member State of notification Spain
(b) Notification number **B/ES/16/05**
(c) Date of acknowledgement of notification 29-04-2016
(d) Title of the project: **Phase II randomized trial of DNX-2401 oncolytic adenovirus added to standard of care for newly diagnosed glioblastoma**

(e) Proposed period of release From September 1st, 2016 until September 1st, 2018

2. Notifier

Name of institution or company: Clínica Universidad de Navarra

3. GMO characterisation

(a) Indicate whether the GMO is a:

viroid		(.)	
RNA virus		(.)	
DNA virus		(X)	
bacterium		(.)	
fungus		(.)	
animal			
-	mammals	(.)	
-	insect	(.)	
-	fish	(.)	
-	other animal	(.)	specify phylum, class

other, specify (kingdom, phylum and class)

(b) Identity of the GMO (genus and species)
Genus: Mastadenovirus
Species: human adenovirus

The GMO DNX2401 (formerly called Delta24-RGD-4C or Delta24-RGD) is an oncolytic adenovirus, which is a replication competent virus selective of tumoral cells. Its genome contains 2 genetic modifications with respect to the wild- type virus (Ad5).

(c) Genetic stability – according to Annex IIIa, II, A(10)

Full genomic sequence analysis and genetic stability analysis of the 24 base pair deletion in the adenoviral E1a region by TaqMan®-based qPCR were done on the Master Viral Bank and on the Purified Filtered Bulk Product. Restriction digestion and molecular weight analysis by loading on an agarose gel for electrophoresis, followed by band size comparison to know molecular weights of marker bands were done on the Master Viral Bank and the Final Vialed Product.
The limit of detection of the qPCR technique was determined to be <15 copies of wild-type Ad5E1a in 3x 10_4 vp/reaction, which is equivalent to less than 1 wild type Ad5E1a copy per 2,000 Ad5-Delta24-RGD (DNX2401) copies. The test was valid with an R_2=0.998 and Ct values for all three negative controls were > 40. No wild type E1a DNA was detected in the filtered final bulk virus test sample analyzed in triplicate (no amplification beyond the threshold fluorescence Ct > 40). A review of the amplification plot indicated that no nascent amplification below the threshold was noted for the three test replicates. Thus, absence of wild type E1a copies, and thus of wild type adenovirus, was confirmed in three independent test samples from the final filtered bulk GMO product, with a 1:2,000 sensitivity.

Adenoviruses have shown to be very stable both in nature and in laboratory. Modified adenoviruses are stable when total genoms do not exceed 105% of the wild type. In this case, is less than 101%.

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?

Yes (.) No (X)

If yes, insert the country code(s)

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?

Yes (X) No (.)

If yes:

- Member State of notification ES
- Notification number B/ES/13/18

Please use the following country codes:
Austria AT; Belgium BE; Germany DE; Denmark DK; Spain ES; Finland FI; France FR; United Kingdom GB; Greece GR; Ireland IE; Iceland IS; Italy IT; Luxembourg LU; Netherlands NL; Norway NO; Portugal PT; Sweden SE

6. Has the same GMO been notified for release or placing on the market outside the Community by the same or other notifier?

Yes (X) No (.)

If yes:

- Member State of notification Netherlands
- Notification number B/NL/08/008

- Member State of notification USA
- Notification number NCT00562003 and NCT00805376

(ClinicalTrials.gov Identifiers)

7. Summary of the potential environmental impact of the release of the GMOs.

The potential environmental impact of the GMO is minor.

It cannot be excluded that the GMO is shed from the primary recipient after brain injection, although it is unlikely. If that happens, the virus could infect the upper respiratory tract cells, as the wild type do.
The infection tropism modification of the GMO is irrelevant, as the wild type is already very efficient in this sense. Importantly, because the GMO has cancer cell specific replication properties it will not replicate in healthy cells of a secondary recipient and further dissemination is thus prevented. Eventual toxicty in secondary receptor cells would be less than the wild type, which is already a mild pathogen.
Theoretically, the GMO could recombine with wild type adenovirus in a recipient infected with both viruses, but that is very unlikely and never observed in practice. The inability of the virus to replicate outside tumor cells, make this recombination highly unlikely. If this would happen a GMO derivative could be formed. Further horizontal transmission of such a theoretical GMO derivative via respiratory tract infections is not expected to be any different from that of wild type adenovirus, causing the same mild symptoms and being self-limiting.

Recombinant variants of Ad5 with other types of adenovirus have not been observed.

Due to the adenoviral specifity for the animal hosts, transmission to species other that human is highly unlikely.

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Adenoviruses do not integrate their ADN in the host, so transmission along the recipient genoma is not possible.

B. Information relating to the recipient or parental organism from which the GMO is derived

1. Recipient or parental organism characterisation:

(a) Indicate whether the recipient or parental organism is a:

(select one only)

viroid		(.)
RNA virus		(.)
DNA virus		(X)
bacterium		(.)
fungus		(.)
animal		
-	mammals	(.)
-	insect	(.)
-	fish	(.)
-	other animal	(.)

(specify phylum, class)

other, specify

2. Name

(i)	order and/or higher taxon (for animals)	Adenoviridae
(ii)	genus	Mastadenovirus
(iii)	species	human adenovirus
(iv)	subspecies	subgroup C
(v)	strain	human serotype 5 (Ad5)
(vi)	pathovar (biotype, ecotype, race, etc.)	…
(vii)	common name	human adenovirus serotype 5 (Ad5)

3. Geographical distribution of the organism

(a) Indigenous to, or otherwise established in, the country where the notification is made:
Yes (X) No (.) Not known (.)

(b) Indigenous to, or otherwise established in, other EC countries:
(i) Yes (X)

If yes, indicate the type of ecosystem in which it is found:

Atlantic	X
Mediteranean	X
Boreal	X
Alpine	X
Continental	X
Macaronesian	X

(ii) No (.)
(iii) Not known (.)

(c) Is it frequently used in the country where the notification is made?
Yes (x) No (.)

(d) Is it frequently kept in the country where the notification is made?
Yes (x) No (.)

4. Natural habitat of the organism

(a) If the organism is a microorganism

water	(.)
soil, free-living	(.)
soil in association with plant-root systems	(.)
in association with plant leaf/stem systems	(.)
other, specify	

The host range of HAd5 is restricted to humans. Although it is also described that chimpanzees (Pan troglodytes), swine (Sus scrofa), cotton rats (Sigmodon hispidus) and some kind of hamsters (Mesocricetus auratus) are semi-permissive species for the replication of human adenovirus, no natural infections with HAd5 have been described in these species to date.

(b) If the organism is an animal: natural habitat or usual agroecosystem:
N/A

5. (a) Detection techniques

The detection of adenoviral particles is done by real- time PCR (RT- PCR), directly from DNA obtained from the tissue/organ tested, and using oligonucleotides that amplify a region of the virus genome. The detection of infectious particles is done by plaque assays, in which the in vitro generation of plaques in monolayers of human cells is examined.

(b) Identification techniques

The specific identification of adenovirus is performed at genomic viral DNA level by PCR using oligonucleotides with a defined complementary sequence and also by restriction enzyme analysis of the purified viral DNA.

6. Is the recipient organism classified under existing Community rules relating to the protection of human health and/or the environment?

Yes (X) No (.)

If yes, specify:
In terms of biosafety, HAd5 are human adenoviruses classified as class 2 biological agents. Adenoviruses are pathogens that can cause human or animal disease but are unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventative measures are available and the risk of spread of infection is limited. Therefore HAd5 manipulation poses a moderate individual risk but low community risk.

7. Is the recipient organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (X) No (.) Not known (.)

If yes:

(a) to which of the following organisms:

humans	(X)
animals	(.)
plants	()
other	(.)

(b) give the relevant information specified under Annex III A, point II. (A)(11)(d) of Directive 2001/18/EC

Over 50 distinct human adenovirus (Ad) serotypes have been isolated, which vary in their pathogenicity. The subgroup C adenoviruses (which includes serotype 5) usually are asymptomatic or cause mild upper respiratory, gastrointestinal and ocular infections in young children. Incubation period is 1-10 days. Shedding via the gut following acute infection can occur, and latent infection of the adenoids and tonsils is also recognised. However, life-long immunity to the specific serotype is thought to be the usual outcome of primary infection. Exposure to subgroup C adenoviruses is widespread in the population; the majority of adults are seropositive for this type of adenovirus. In immunecompetent adults, infections with Ad5 are mostly asymptomatic and usually self-limiting.
Under normal circumstances other animals than humans are not susceptible to human serotype adenoviruses and no diseases in other animals can be demonstrated caused by the human serotype. Adenoviruses do not integrate genetic material in the host.
Injection of adenovirus in testes in animal models, have failed to produce transgenic animals.

8. Information concerning reproduction

(a) Generation time in natural ecosystems:
Ad5 is not found in natural ecosystems. Adenovirus serotype 5 replicates in approximately 24 hours in cancer cells.

(b) Generation time in the ecosystem where the release will take place:
In cancer cells, the GMO replicates with a generation time similar to that of wild type Ad5. In healthy cells, its replication capacity is strongly inhibited.

(c) Way of reproduction: Sexual .. Asexual ..
Not applicable

(d) Factors affecting reproduction:
The reproduction of the GMO is restricted compared to wild type Ad5 due to the Delta24 modification in the E1A region. This modification abrogates the capacity of adenovirus encoded E1A protein to bind pRB pocket protein family members. This prevents release of E2F from E2F-pRB complexes, which in turn prevents activation of adenoviral genes and genes involved in DNA synthesis and progression through cell cycle. Consequently, the GMO is incapable of inducing host cells to pass the
G2/M checkpoint and can therefore replicate only in cells with a defective pRb pathway. A defective pRb pathway is a hallmark of cáncer, and pRb defects have not been described to occur in healthy cells. Consequently, the GMO exhibits strongly reduced replication property compared to wild type Ad5 in quiescent normal human cells.

9. Survivability

(a) ability to form structures enhancing survival or dormancy:

(i) endospores (.)
(ii) cysts (.)
(iii) sclerotia (.)

(iv)	asexual spores (fungi)	(.)
(v)	sexual spores (funghi)	(.)
(vi)	eggs	(.)
(vii)	pupae	(.)
(viii)	larvae	(.)
(ix)	other, specify	

Not has this ability

(b) relevant factors affecting survivability:

Adenoviruses rapidly lose their bioactivity at room temperature. HAd5 is susceptible to 1% sodium hypochlorite, 2% glutaraldehyde and sodium dodecyl sulfate (SDS) 0.25%. And it is also sensitive to heat (> 56 °C). Inactivation of HAd5 is best achieved by autoclaving at 121ºC for 15 minutes; also conventional disinfectants (bleach, soap or similar) are effective against HAd5 if kept in contact for 20-30 min. In frozen condition, the virus can be kept for years.

10. (a) Ways of dissemination

The common route of Ad5 transmission is via inhalation or uptake in the eye of aerosols produced through couching/sneezing by infected individuals. Under moist conditions, anc close proximity, transmission is effective. Adenovirus transmission can also occur via the fecal-oral route, but this transmission route requires intimate contact and is unusual. Infection of casual contacts after enteric administration is unlikely, even with fully virulent adenoviruses. Besides humans and chimpanzees, there is no effective infection of other hosts.

(b) Factors affecting dissemination

Dissemination is affected by the dose shed, the production of aerosols and the intimacy of contact.

11. Previous genetic modifications of the recipient or parental organism already notified for release in the country where the notification is made (give notification numbers)
Not applicable.

C. **Information relating to the genetic modification**

1. Type of the genetic modification

(i)	insertion of genetic material	(X)
(ii)	deletion of genetic material	(X)
(iii)	base substitution	(.)
(iv)	cell fusion	(.)
(v)	others, specify	

2. Intended outcome of the genetic modification

The GMO contains a full-length Ad5 genome with the following two modifications. First, a 24 base pair deletion (Delta24) was introduced in the pRb-binding CR2 domain of the E1A gene. This modification renders adenovirus replication defective in normal cells with

intact Rb protein. As a result, the GMO exhibits selective replication in cells with a defective pRb pathway, most notably cancer cells. Second, a short sequence encoding an integrin-binding RGD-4C peptide was inserted in the fiber gene. This modification allows cell infection via high-affinity binding via several types of integrins. As a result, also cells that do not express high-affinity receptors for the virus and are therefore difficult to infect with Ad5, but that do express these integrins can be infected with the GMO. Glioblastoma multiforme cells in brain tumors exhibit the characteristics (pRb pathway defects; low adenovirus receptor expression, high integrin expression) for which the GMO was designed. The GMO is intended to replicate efficiently and selectively in glioblastoma multiforme cells in the brain of patients injected with the GMO, thereby killing these cancer cells effectively.

3. (a) Has a vector been used in the process of modification?

Yes (X) No (.)

If no, go straight to question 5.

(b) If yes, is the vector wholly or partially present in the modified organism?

Yes (X) No (.)

If no, go straight to question 5.

4. If the answer to 3(b) is yes, supply the following information

(a) Type of vector

plasmid	(X)
bacteriophage	(.)
virus	(.)
cosmid	(.)
transposable element	(.)
other, specify	

(b) Identity of the vector

The GMO is made from the plasmid vector pVK526 (Suzuki et al., Clin. Cancer Res. 7, 120-126, 2001). This plasmid contains a bacterial origin of replication, an ampicillin resistance gene and a full-length Ad5 genome with the following two modifications:

- a 24 base pair deletion (Delta24) in the pRb-binding CR2 domain of the E1A gene.
- a short synthetic sequence insert encoding an integrin-binding RGD-4C peptide in the fiber gene.

(c) Host range of the vector

The plasmid vector replicates in E. coli bacteria.

(d) Presence in the vector of sequences giving a selectable or identifiable phenotype

Yes (X) No (.)

antibiotic resistance	(X)
other, specify	

Indication of which antibiotic resistance gene is inserted

Ampicillin

(e) Constituent fragments of the vector
see C 4 b

(f) Method for introducing the vector into the recipient organism

(i)	transformation	(.)
(ii)	electroporation	(.)
(iii)	macroinjection	(.)
(iv)	microinjection	(.)
(v)	infection	(.)
(vi)	other, specify:	

Upon release of the Ad5-derived DNA genome from the plasmid vector by restriction enzyme digestion, it is transfected into A549 human epithelial lung carcinoma cells. In these susceptible cells, the GMO is formed. The GMO contains only the full-length Ad5 genome with the two modifications. The other components of the plasmid vector are not transferred into the GMO.

5. If the answer to question B.3(a) and (b) is no, what was the method used in the process of modification?

(i)	transformation	(.)
(ii)	microinjection	(.)
(iii)	microencapsulation	(.)
(iv)	macroinjection	(.)
(v)	other, specify	(.)

6. Composition of the insert

(a) Composition of the insert

Synthetic DNA sequence encoding a cyclic RGD-4C motif.

(b) Source of each constituent part of the insert
Synthetic

(c) Intended function of each constituent part of the insert in the GMO
Extension of infection range to include human cells with low expression of high-affinity adenovirus receptor, but high integrin expression.

(c) Location of the insert in the host organism

- on a free plasmid (.)
- integrated in the chromosome (.)
- other, specify: integrated in the fiber gene ORF on the virus DNA

(d) Does the insert contain parts whose product or function are not known?
Yes (.) No (X)

If yes, specify

D. Information on the organism(s) from which the insert is derived

1. Indicate whether it is a:

viroid (.)
RNA virus (.)
DNA virus (.)
bacterium (.)
fungus (.)
animal
- mammals (.)
- insect (.)
- fish (.)
- other animal (.)
(specify phylum, class)

other, specify: synthetic DNA (D 2 – D 5 are not applicable)

2. Complete name

(i) order and/or higher taxon (for animals) …
(ii) family name for plants …
(iii) genus …
(iv) species …
(v) subspecies …
(vi) strain …
(vii) cultivar/breeding line …
(viii) pathovar …
(ix) common name …

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?
Yes (.) No (.) Not known (.)
If yes, specify the following:

(b) to which of the following organisms:

humans (.)
animals (.)
plants (.)
other ..

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism
Yes (.) No (.) Not known (.)

If yes, give the relevant information under Annex III A, point II(A)(11)(d):
…

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?

Yes (.) No (.)

If yes, specify

5. Do the donor and recipient organism exchange genetic material naturally?

Yes (.) No (.) Not known (.)

E. Information relating to the genetically modified organism

1. Genetic traits and phenotypic characteristics of the recipient or parental organism, which have been changed as a result of the genetic modification

(a) is the GMO different from the recipient as far as survivability is concerned?

Yes (.) No (X) Not known (.)

Specify

(b) is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned?

Yes (X) No (.) Unknown (.)

Specify:

Due to the Delta24-modification, the GMO cannot replicate in quiescent normal cells with intact Rb pathway. Thus, in healthy cells the reproduction of the GMO is reduced compared to the parental virus Ad5. The RGD-4C modification broadens the infection-tropism of the virus. Also cells that do not express high-affinity adenovirus receptors and are therefore difficult to infect with Ad5, but do express certain types of integrins can be infected with the GMO. This modification could thus potentially increase the reproduction of the GMO compared to wild type Ad5. Notably, the broadening of infection by the RGD-4C modification does not affect the attenuation of replication by the Delta24 modification. Normal cells lacking high-affinity adenovirus receptors but expressing integrins, which would not be infected efficiently by wild type Ad5, can be infected by the GMO, but the GMO will not replicate in these cells if they are non-dividing. Therefore, the RGD-4C modification is considered not to increase the reproduction rate. Overall, the reproduction of the GMO is reduced compared to the parental virus Ad5.

(c) is the GMO in any way different from the recipient as far as dissemination is concerned?

Yes (X) No (.) Not known (.)

Specify:

Horizontal transmission of the GMO may occur via the natural Ad5 transmission route, i.e., uptake of aerosols created by coughing or sneezing via inhalation or the eye. Since this uptake is already very efficient via the natural Ad5 receptors, the RGD-4C modification in the GMO is irrelevant in this respect. In contrast, the Delta24 modification attenuates virus replication in normal cells, thus reducing further spread of the GMO from secondary recipients. Thus, if the GMO is released into the environment, it is expected to transmit

horizontally to secondary recipients like wild type Ad5, but due to its attenuated replication property it will extinguish, thus reducing further dissemination.

(d) is the GMO in any way different from the recipient as far as pathogenicity is concerned?

Yes (X) No (.) Not known (.)

Specify:

Due to the Delta24-modification, the GMO cannot replicate in quiescent normal cells with intact Rb pathway. Thus, the replication-tropism of the GMO is narrowed compared to the parental virus Ad5. The already low pathogenicity of wild type Ad5 is therefore further reduced. The RGD-4C modification broadens the infection-tropism of the virus. Also cells that do not express high-affinity adenovirus receptors and are therefore difficult to infect with Ad5, but do express certain types of integrins can be infected with the GMO. This modification could thus potentially increase the pathogenicity of the GMO compared to wild type Ad5. Following high-dose systemic administration to cotton rats, the GMO did not show altered biodistribution compared to a control virus lacking the RGD-4C infection-tropism modification. A clinical trial done in ovarian cancer with intraperitoneal delivery of the virus did not find changes in biodistribution. Thus, apparently cells that do not express natural Ad5 receptors in sufficient amounts to allow effective infection, but that do express integrins to which RGD-4C can bind to mediate virus uptake are not detectably reached in steady-state healthy tissues.

The GMO is therefore not considered more pathogenic than wild type Ad5 to steady-state healthy cells and tissues that can be reached via the circulation.

Notably, the broadening of infection by the RGD-4C modification does not affect the attenuation of replication by the Delta24-modification. Normal actively dividing cells lacking high-affinity adenovirus receptors but expressing integrins, which would not be infected efficiently by wild type Ad5, can be infected by the GMO. However animal studies and clinical trial using this OMG have demonstrated that there is no infection in the normal actively dividing cells.

2. Genetic stability of the genetically modified organism

The GMO is stable. The GMO is produced in the A549 cell line. The MCB was tested and found negative for a range of viral contaminants, including Ad5 by quantitative PCR for E1A and hexon genes. Complementation by or recombination with wild type E1A during propagation of the GMO on A549 cells can therefore be excluded. Full genomic sequence analysis and genetic stability analysis of the 24 base pair deletion in the adenoviral E1A region by TaqMan®-based qPCR were one on the Master Viral Bank and on the Purified Filtered Bulk Product. Absence of wild type E1A copies, and thus of wild type adenovirus, was confirmed in three independent test samples from the final filtered bulk GMO product, with a 1:2,000 sensitivity (see A 3 c).

The GMO has a genoma size less than101% of the wild type virus; adenovirus have been found to be genetically stable when genoma size do not exceed 105%.

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?

Yes (X) No (.) Unknown (.)

(a) to which of the following organisms?

humans	(X)
animals	(.)
plants	(.)
other	(.)

(b) give the relevant information specified under Annex III A, point II(A)(11)(d) and II(C)(2)(i)

Point II (A) 11)(d)

Over 50 distinct human adenovirus (Ad) serotypes have been isolated, which vary in their pathogenicity. The subgroup C adenoviruses (which includes serotype 5) usually are asymptomatic or cause mild upper respiratory, gastrointestinal and ocular infections in young children. Incubation period is 1-10 days. Shedding via the gut following acute infection can occur, and latent infection of the adenoids and tonsils is also recognised. However, life-long immunity to the specific serotype is thought to be the usual outcome of primary infection. Exposure to subgroup C adenoviruses is widespread in the population; the majority of adults are seropositive for this type of adenovirus. In immunecompetent adults, infections with Ad5 are mostly asymptomatic and usually self-limiting.

Under normal circumstances other animals than humans are not susceptible to human serotype adenoviruses and no diseases in other animals can be demonstrated caused by the human serotype. Adenovirus do not integrate genetic material in the host.

Injection of adenovirus in testes in animal models, have failed to produce transgenic animals.

Point II(C)(2)(i)

The GMO will be administered to the brain. The main physiological process expected to take place there is lytic replication in and destruction of cancer cells. Normal brain cells are post-mitotic. Due to its replication attenuation in non-cancerous cells, the GMO is expected to be less pathogenic in this locale than the mild pathogen Ad5. However, leakage of the GMO from the brain is possible to the cerebrospinal fluid and/or the blood. The likelihood for this to occur is unknown. Therefore, we discuss the worst case scenario, assuming that this will happen. It is expected that – similar to what is known for Ad5 - most of the GMO that may have leaked into the circulation will be sequestered and destructed by Kupffer cells in the liver. The GMO could have ability ot infect cells usually not infected by the wild type, even if this happens the replication ability would be limited in non tumor cells. A biodistribution study following intravenous injection in cotton rats where the GMO was compared to a control virus lacking the RGD-4C modification revealed almost identical distributions for the two viruses over blood and tissues. A clinical study using the virus intraperitoneally also failed to find altered biodistribution in humans. This suggested that the impact of the RGD-4C modification on the spreading of the virus through the body is minimal.

Due to the reduced replication ability, colonization by the virus seems improbable.

4. Description of identification and detection methods

(a) Techniques used to detect the GMO in the environment

Presence of the GMO DNA is detected by specific, quantitative PCR, using an

amplification primer pair specific for the adenovirus fiber gene and a TaqMan® fluorescent probe specific for the RGD-4C encoding insert in the fiber gene. Functional assays for viable virus, which do not discriminate between the GMO and wild type adenovirus, include an infection assay and a plaque assay. In these assays, a cell line susceptible to adenovirus infection and replication is exposed to serial dilutions of the virus. In the infection assay, the cells are stained for adenovirus hexon protein expression and results are expressed as infectious units per ml. In the plaque assay, the cells are cultured until viral plaques are visible. The plaques are counted and the results are expressed as plaque forming units per ml.

To confirm the identity of the GMO in the functional assay, the quantitative PCR can be done on plaque-containing cultures.

(b) Techniques used to identify the GMO

Specific quantitative PCR described above (D.4.a).

F. Information relating to the release

1. Purpose of the release (including any significant potential environmental benefits that may be expected)

Malignant brain tumors, especially glioblastoma multiforme (GBM) are among the most aggressive human tumors that exist. The median survival after diagnosis is approximately one year, despite maximal aggressive treatment. Novel therapies for this devastating disease are urgently warranted, due to an unmet need for an effective therapy. One of the key problems in GBM treatment is its invasive character, which causes the tumor to consist of two components: a solid component that can be safely resected with high precision neurosurgical techniques; and a diffuse component, consisting of infiltrated tumor cells in the surrounding healthy brain, inaccessible for surgery. Due to the existence of the Blood-Brain Barrier, drug-based therapies such as chemotherapy remain insufficiently effective. The GMO is a genetically modified adenovirus with strong anti-tumor efficacy due to its capability of selective replication in cancer cells, resulting in direct and very efficient cell kill (tumor cell lysis). Because the expression level of high-affinity adenovirus receptors is usually low on glioma cells, the GMO incorporates an integrin binding peptide (RGD- 4C peptide) in its fiber capsid protein. This peptide binds with high affinity to several types of integrins, especially type V integrins, abundant on the surgafe of glioma cells. Infiltrated brain tumor cells around the solid brain tumor mass show high integrin expression, whereas in surrounding normal brain integrin expression is absent. The GMO will be delivered to infiltrating tumor cells in the diffuse part of the tumor in patients with GBM during surgery, using intraparenchimal inyection after biopsy or tumor resection of the solid tumor. The clinical trial aims to acquire clinical safety and efficacy data with the GMO plus standard radiotherapy and chemotherapy. The ultimate aim is to develop an effective treatment against GBM. The same GMO was used in the past years 2013-2015 on a different clinical trial in our institution that showed safety and some hints of efficacy in recurrent GBM.

2. Is the site of the release different from the natural habitat or from the ecosystem in which the recipient or parental organism is regularly used, kept or found?

Yes (.) No (X)

If yes, specify

3. Information concerning the release and the surrounding area

(a) Geographical location (administrative region and where appropriate grid reference):
Clínica Universidad de Navarra, Pamplona, Navarra, Spain.

(b) Size of the site (m^2):
(i) actual release site (m^2): Operating room 40 m^2
The virus will be keep at –80ºC in the hospital pharmacy and the administration will be made by the neurosurgeons in the operating room.

(ii) wider release site (m^2):
Clínica Universidad de Navarra

(c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:
Not applicable

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO

Not applicable.

4. Method and amount of release

(a) Quantities of GMOs to be released:
Patients will receive a single infusion of 5 $x10^{10}$ viral particles in 1mL.

(b) Duration of the operation:
Virus infusion will take 15 minutes approximately.

(c) Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of the release

Sealed and duly labeled vials containing DNX2401 will be supplied for clinical use to the pharmacy department. The study medication will be store in a –80ºC freezer within an area with restricted access (pharmacy personnel only). The storage and handling of the virus prior to administration will be the pharmacist´s responsability. The dose will be load into 1 ml syringe under aseptic conditions following biosafety level 2 practices and equipment (safety cabinet: Class II, type B) and according to local GMO requirements. All medication left and material used for drug preparation will be destroyed locally in line with the institution´s destruction procedures. The safety cabinet will be disinfected before and after its use with a conventional disinfectant, following the standard cleaning procedures of the hospital. The pharmacist will supply the medication to the Principal Investigator/Co-Investigator in a in an airtight container, according to policies for transport of live virus vaccines, that will be closed and labelled with relevant information such as product code and "contains genetically modified organism (GMO)" and "Biohazard product". Pharmacy department and Operating room area are in the same floor, with a direct acces, therefore transporting risks are minimal.

The release will be responsibility of trained medical staff, in accordance with the clinical protocol and the Good Clinical Practices, in an operating room. The medical staff must wear disposable lab coat, gloves, glasses, and FFP3 masks. Before the administration, the operating room will be closed and only the necessary personnel will be allowed inside. All the personnel inside the room will be medical staff or nurses and will be trained in the procedure. A warning sign on the door will inform about the special procedure and restrictions and potential risks, and type III medical waste containers will be placed inside the operating room.

All the material in contact with the GMO (needles, gloves, empty vials, etc.) will be considered as a type III medical waste (biological/biohazardous) and will be disposed accordingly. It will be disposed off, in specific Type III biohazardous waste containers that will be duly labeled and managed by a specialized company to be processed and destroyed under the protocol already in place in the institution for this kind of waste.

After the administration, all equipments and working surfaces in possible contact with the agent will be disinfected using Limoseptic® (glutaraldehyde 2,5%) and Desinfect® (sodium hypochlorite 5%). The rest of the room will be cleaned following the stardard protocol of our hospital named as "last OR cleaning" (which includes Limoseptic® and Desinfect®).

Once DNX2401has been administered, the patient will be hospitalized and monitored at least for the next 24h. The room will be supervised by trained nurses. It will keep the warning sign on the door, which will remain closed. The patient visits will be restricted and it will be mandatory that they wear disposable lab coat, gloves and mask.

Once the patient is discharged the hospital room, will be disinfected following the stardard protocol of our hospital (which includes Limoseptic® with glutaraldehyde 2,5%).

Patients and family members will receive preventive measures instructions, including basic hygienic measures, to avoid the spread of the GMO beyond the site of release. The outpatient will be counseled prior to release, to avoid public areas, public transportation, young children, the aged, pregnant women, and immunosuppressed, for 3 days after the adenoviral administration.

5. Short description of average environmental conditions (weather, temperature, etc.):
Hospital environment, controlled conditions.

6. Relevant data regarding previous releases carried out with the same GMO, if any, specially related to the potential environmental and human health impacts from the release.

Four clinical trial have been conducted with the same GMO and no impacts to the environment not to the human health have been reported, two more are opened now:

Phase I study conducted in the USA (University of Alabama in Birmingham), the DNX2401was administered to patients with recurrent malignant ginecologic diseases once a day for three days by intraperitoneal infusion. Total dose was 3×10^{12} , 100 higher than planned in this trial. No severe toxicity related to the virus administration was observed. No environmental impact of the release was observed. Viral ADN was analyzed by PCR in the patient fluid, no viral ADN was detected in patients recieving dose similar to the dose to be used in the present trial (3×10^{10}) or less; insigniffican amount of viral ADN was detected in a few patients in the higher dose cohort.

Phase I trial with the DNX2401administered to recurrent GBM tumors was completed in the USA (MD Anderson Cancer Center, Houston, Tx study number ID01-310; Clinical Trial.gov NCT00805376). In this trial the virus is injected in the brain in the Operating room; included and exhaustive determination of virus shedding, it reached the maximum cohort dose, 1×10^{11}, without observing any significant shedding of the GMO from the patient. Patients go home within a few days after virus infusion and return regularly for analysis. No impact on the enviroment has been noted.

Phase I trial with DNX2401administered to recurrent GBM tumors in Rotterdam, Netherlands, in Erasmus Medical Center, release notification number B/NL/08/008, (Clinical Trial.gov NCT01582516). In this trial patients virus infusion in the brain took place within a period of 50-68 hours, depending on the number of intracranial catheters used for infusion. This trial started in 2010, and includes and exhaustive determination of virus presence in body fluids it reached the maximum cohort dose, 10×10^{11}, without observing any significant shedding of the OM from the patient. No relevant data regarding environmental or human health impacts have been reported.

Phase I trial was completed in our institution, Clinica Universidad de Navarra, from October 2013 to November 2015. Administration protocol was essentialy the same of the trial proposed here. 31 patients were injected. No relevant toxicity related to the virus injection was noticed. No case of transmission, or unexplained disease related to virus, was observed.

G. Interactions of the GMO with the environment and potential impact on the environment, if significantly different from the recipient or parent organism

1. Name of target organism (if applicable)

(i)	order and/or higher taxon (for animals)	Primate
(ii)	family name for plants	Hominidae
(iii)	genus	Homo
(iv)	species	Homo sapiens
(v)	subspecies	Homo sapiens sapiens
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	…

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)

The GMO is anticipated to infect brain tumor cells in the brain of GBM patients, to replicate selectively in these cells and to kill these cells by lysis. Lysed cancer cells will release GMO progeny virus that can also infect and kill brain tumor cells. The main objective of the trial is to investigate the efficacy of administering the GMO to the brain of patients with newly diagnosed GBM.

3. Any other potentially significant interactions with other organisms in the environment.

There is a theoretical, but highly unlikely, risk that the GMO and a wild type adenovirus infect the same human cell and recombine to form a new GMO with wild type replication properties and expanded (RGD-4C – integrin interaction mediated) tropism. This newly formed GMO might be less harmless than wild type adenovirus.

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?

Yes (.) No (X) Not known (.)

Give details

The GMO has strongly decreased competitiveness compared to wild type adenovirus due to the Delta24-modification in the E1a gene that attenuates replication in non-cancerous cells. Notably, the broadening of infection by the RGD-4C modification does not affect the attenuation of replication by the Delta24-modification. The ability to infect new organism of the wild type depends on the high infection tropism for the cells of the upper respiratory pathways. The modification of the fiber increase the capacity to infect cells with high leves of integrin expression, which are if present, few in the upper respiratory pathways, so it do not provide an advantage for host infection. Thus, changed infection profile does not compensate for the loss of competitiveness of the GMO due to defective replication in non-cancerous cells.

5. Types of ecosystems to which the GMO could be disseminated from the site of release and in which it could become established

According to the proposed conditions for the GMO release it is highly unlikely that Delta24-RGD interacts with any untargeted population. Being the host range of Ad5 restricted to humans, and that the genetic modifications contained in DNX2401do not modify the species specificity, the likelihood of productive transmission to animals is negligible. And in the unlikely event of unwanted administration to untargeted population, the intrinsic selectivity would avoid dissemination to nontumour cells.

6. Complete name of non-target organisms which (taking into account the nature of the receiving environment) may be unintentionally significantly harmed by the release of the GMO

Not applicable

(i)	order and/or higher taxon (for animals)	…
(ii)	family name for plants	…
(iii)	genus	…
(iv)	species	…
(v)	subspecies	…
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	…

7. Likelihood of genetic exchange in vivo

(a) from the GMO to other organisms in the release ecosystem:
Exchange between the GMO and other organisms is highly unlikely (see G3).

(b) from other organisms to the GMO:
Exchange between the GMO and other organisms is highly unlikely (see G3).

(c) likely consequences of gene transfer:
Transfer of wild type E1A to the GMO or transfer of the RGD-4C modification to wild type adenovirus would create a new GMO with wild type replication properties and changed infection tropism. Infection with such a theoretical GMO will probably be self-

limiting due to existing immunity against adenovirus and its lack of competitive advantages.

8. Give references to relevant results (if available) from studies of the behaviour and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.):
N/A

9. Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)
N/A

H. Information relating to monitoring

1. Methods for monitoring the GMOs

After the inyection of the virus, the patient will be monitored including collecting any adverse event at least until 18 weeks after vitus injection. This period is much longer the time the wild type adenovirus infection persists. Any adverse even will be notified following the clinical trial protocol. After the fist 18 weeks, the follow up will be less frequent, incluiding 30 months after the release of the OMG, following the clinical trial protocol.

Clinical trials performed in MDACC using the same GMO has proved that the shedding of the Delta24-RGC-4C was very low and has no effect on the environment. The paper published in 2010 about the clinical trial in patients with recurrent malignant ginecologic diseases performed viral shedding studies that showed insignificant shedding in the serum, saliva, and urine (Kinball K et al, *Clin Cancer Res* 2010), appearing only at doses 100 times bigger than the dose of this trial. In the clinical trial with GBM in Erasmus Medical center, similar methods for monitoring the GMO were performed and showed that the presence of Delta24-RGD-4C in the serum, saliva and urine was also insignificant, with higher doses of virus that will be used in our trial. Having this information, the monitoring in this trial will be based on frequent clinical evaluation of the patient.

The security advices for patients out-hospital will include conseling about strict personal hygiene, frequent hand washing and avoiding the contact with vulnerable population, as childs or inmunosuppressed patients, as well of early reporting any upper respiratory disease symptoms.

2. Methods for monitoring ecosystem effects

Based on the low probability of transmission to thirds and the inability of DNX2401to amplify or replicate in any other species, and taking into consideration the controlled conditions for the GMO release, the likelihood of any environmental impact due to the shedding to untargeted population is considered negligible.

There is no risk for shedding of virus into the ecosystem. Therefore, ecosystem effects will not be monitored.

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms

Not applicable. The stable transfer of genetic material to any other species different to human is not possible. Additionally, the likelihood of interaction between the GMO and any untargeted population is negligible due to the limited environmental shedding under the proposed conditions of the release, the transitory presence of DNX2401genomes in non-human populations (based in the specie-specificity of human adenoviruses) and the replication selectivity of DNX2401for tumour cells.

4. Size of the monitoring area (m^2)

Operating room during the virus adminsitration, 40 m^2
Intensive Unit Care room for hospitalization room for the first 24 hs and room hospital for the next day: 30 m^2
Exploration room for next visits: 20 m^2

5. Duration of the monitoring

After GMO administration a very close follow-up will be performed during 18 weeks, which is much longer than adenoviral infection persists. Any adverse even will be notified in agreement with the clinical trial protocol.

6. Frequency of the monitoring

The follow up includes clinical evaluation, phisycal and neurological exams every 2 weeks or when the patien is symptomatic, and blood test every 2 weeks. After the first 18 weeks, the follow up will be less frequent, until 30 months or after death.

I. Information on post-release and waste treatment

1. Post-release treatment of the site

After the administration, all equipments and working surfaces in possible contact with the agent will be disinfected using Limoseptic® (glutaraldehyde 2,5%) and Desinfect® (sodium hypochlorite 5%). The rest of the room will be cleaned following the stardard protocol of our hospital named as "last OR cleaning" (which includes Limoseptic® and Desinfect®).

Once the patient is discharged, the hospital room, will be disinfected following the stardard protocol of our hospital (which includes Limoseptic® with glutaraldehyde 2,5%).

2. Post-release treatment of the GMOs

Treatment of the GMO is not required.

3. (a) Type and amount of waste generated

Waste includes the syringes used for virus administration, and the viral continer, wound-dressings, bed linen, disposable cleaning cloths, disposable clothing worn by the patient, disposable crockery, personnel and visitors, etc; and excreta and secreta of patients.

3. (b) Treatment of waste

All the material in contact with the GMO (needles, gloves, empty vials, etc.) will be considered as a type III medical waste (biological/biohazardous) and will be disposed accordingly. It will be disposed off, in specific Type III biohazardous waste containers that will be duly labeled and managed by a specialized company to be processed and destroyed under the protocol already in place in the institution for this kind of waste.

After the administration, all equipments and working surfaces in possible contact with the agent will be disinfected using Limoseptic® (glutaraldehyde 2,5%) and Desinfect® (sodium hypochlorite 5%) or autoclaved.

J. Information on emergency response plans

1. Methods and procedures for controlling the dissemination of the GMO(s) in case of unexpected spread.
In the event of spreas of the agent, all the personnel present should protect with FFP· masks, gloves, glasses and disposable gown. The doors of the room will be closed and nobody should enter until the cleaning is finished.Spilled GMO will be wiped up with dry tissues. Subsequently, the surface will be disinfected with tissues soaked in active chlorine solution (Desinfect®, sodium hypochlorite 5%). All collected materials and gloves worn during execution of the cleaning procedure are disposed according to procedures for type III bio-hazardous material.

2. Methods for removal of the GMO(s) of the areas potentially affected

Spilled GMO will be wiped up with dry tissues. Subsequently, the surface will be disinfected with tissues soaked in active chlorine solution (Desinfect®, sodium hypochlorite 5%). All collected materials and gloves worn during execution of the cleaning procedure are disposed according to procedures for type III bio-hazardous material.

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread
Not applicable.

4. Plans for protecting human health and the environment in the event of an undesirable effect

Patients will be monitored for the occurrence of serious adverse events (SAE) according to the clinical protocol: each SAE will be registered and evaluated, and health authorities will be notified when relevant. Specific plans for protecting the environment are not considered necessary, for the reasons stated above.