1. Publishable Summary

1.1. THERADPOX Objectives and partners involved

Cancer is today the second cause of disease-induced mortality worldwide. Among innovative treatments for cancer, virotherapy holds great promise. Oncolytic viruses (OVs) are replicating micro-organisms (viruses) that have been selected or engineered to grow inside and kill tumour cells. It is well known that as a tumour evolves, mutations in multiple genes contribute to the malignant phenotype. Oncolytic viruses specifically target cancer cells because they are able to exploit the very same cellular defects that promote tumour growth.

Over the past century, a series of case studies and anecdotal reports have indicated that viruses could indeed be used as anticancer therapeutics. However, it has only been in the last decade, as we have gained a more comprehensive understanding of the molecular basis of tumour genesis, that it has been possible to develop the oncolytic virus therapeutic platform.

In principle, oncolytic viral therapy offers several advantages over conventional anticancer drugs. Viruses can be rapidly modified by recombinant DNA technology, allowing for the rational creation of 'designer viruses'. Addition of genes that code for toxic payloads or immune-stimulatory products, as well as the natural propensity of the virus to promote tumour-specific inflammation, makes oncolytic viruses multi-modal therapeutics. Owing to their ability to self-replicate within the cancer cell, oncolytic viruses have unique pharmacokinetic properties that are distinct from conventional therapeutics. In a sense, oncolytic viruses can be thought of as miniature biological machines that we can program to specifically target, replicate in and ultimately kill tumour cells. The arsenal of oncolytic viruses that are under development is ever expanding, as are the creative strategies for achieving tumour-specific replication. Owing to the complexities of tumour biology and the heterogeneity of human tissues, it seems unlikely that one kind of virus or a single targeting strategy will be sufficient to treat all cancers.

Although the number of different types of oncolytic viruses that have been tested in preclinical trials is increasing, only a few have made the transition into the clinic. Only recently, the first patients were treated in the first bona fide clinical trials of oncolytic virus therapy. Although more than 50 phase I or II clinical trials have been subsequently conducted, there is only a single published phase III trial. Thus, our clinical experience is limited — we are still studying these new therapeutics and developing ways to optimize their efficacy.

The THERADPOX project focused on specific oncolytic vectors such as pox virus and adenovirus based vectors, owing to their inherent strong oncolytic potency and safety record.

The OVs derived from these viral platforms are described below:

- *Vaccinia virus (VV):* the advantages of VV as OV are: a quick and efficient life cycle, strong lytic activity and rapid cell-to-cell spreading. The virus can infect a wide variety of human tissues but does not cause any known human disease. VV was the first widely used vaccine which resulted in the eradication of smallpox on earth. As a result, the responses, safety profile, adverse reactions have been extensively studied and documented.
- *Myxoma virus (MV):* MV causes myxomatosis in European rabbits but is non-pathogenic in man. However, *Myxoma* virus productively infects a variety of human tumour cells (Sypula et al., 2004), suggesting a significant potential for exploiting MV as a novel OV platform.
- *Adenovirus (Ad):* one of the most extensively studied viruses which have been engineered for gene therapy and for virotherapy of cancer, particularly serotypes 2 (Ad2) and 5 (Ad5). The virus is endemic in the human population and its natural pathogenicity is associated with mild respiratory infections (common cold). It can be grown easily and to high titers and the methodology to generate recombinant viruses is well established.

Through the multidisciplinary Work Plan proposed and the strong and complementary expertise of the 10 European partners involved, the THERADPOX project participated in:

- i) Generating advanced knowledge which could be translated towards a safer cancer treatment with an increased therapeutic index,
- ii) Leading to the proposal of new guidelines and standards for the development of OVs, and
- iii) Fostering the competitiveness of Europe in the war against cancer.

The consortium includes 10 European partners (see Table 1 below) with expertise in oncolytic pox viruses and adenoviruses, preclinical animal model systems, improved delivery systems, application software development and standardization of data management and general consortium management.

Table 1: THERADPOX Consortium

Part n°	Organisation name	Organisation short name	Scientific Team Leader	Expertise	Country
1	TRANSGENE S.A.	**TRANSGENE**	Monika LUSKY	Coordinator	**FR**
2	Institut Català d'Oncologia	**ICO**	Ramon ALEMANY	Oncolytic adenovirus targeting de-regulated E2F pathway	**ES**
3	Institut National de la Recherche Agronomique	**INRA**	Stéphane BERTAGNOLI	*Myxoma* virus	**FR**
4	University of St Andrews	**USTAN**	Richard IGGO	Oncolytic adenovirus targeting colon cancer	**UK**
5	DelSiTech Ltd	**DELSITECH**	Harry JALONEN	Controlled delivery systems	**FI**
6	Helsingin yliopisto	**UH.RDD**	Akseli HEMMINKI	Oncolytic adenovirus with enhanced tumour infectivity non-invasive imaging	**FI**
7	NewLab	**NEWLAB**	Mohammed BENBOUCHAIB	Software development	**FR**
8	Paul Ehrlich Institut	**PEI**	Gerd SUTTER	Oncolytic *Vaccinia* virus	**DE**
9	Oncotest GmbH	**ONCOTEST**	Michael HOFMANN	Preclinical patient relevant animal models	**DE**
10	ACIES	**ACIES**	Frédéric CRUTEL	Management	**FR**

The THERADPOX project aimed at engineering novel, safe and optimised Oncolytic Vectors (OVs) with an increased therapeutic index: pox viruses (*Myxoma* virus (MV), Copenhagen *Vaccinia* virus (VVCop) and adenoviruses (Ad) for the therapy of colorectal, pancreatic and ovarian cancers.

THERADPOX OVs were engineered to:

- Selectively target cancer cells *in vivo* (specifically target colorectal, pancreatic and ovarian cancer cells,)
- Selectively replicate and propagate in tumour cells *in vivo*
- Be armed with therapeutic genes rendering only tumour cells sensitive to chemotherapy *in vivo*: OVs were engineered to express in the infected cancer cells the pro-drug converting enzyme FCU1 (converting the non-toxic pro-drug 5FC to the toxic end-product 5FU) enabling the conversion of a non-toxic molecule (given orally or intravenously) to toxic compounds only in tumour cells
- Widely spread within tumours *in vivo* to permit total tumour eradication.

1.2. Main achievements of the THERADPOX project

THERADPOX OVs were assessed for safety and non pathogenic effects in normal tissue and for efficacy *in vitro* in a panel of cancer cell lines (from the selected tumour models) and the selected tumour models *in vivo* in tumour bearing animals. In addition, an original controlled delivery system was developed to increase the half-life of vectors (time necessary for one-half of the original amount of viruses to be eliminated) in the circulation and to lower potential systemic toxicological side-effects. Moreover, a software application for fast, sharp, pertinent and standardised data analysis and interpretation was also developed within the consortium and distributed among partners.

Towards these goals, the following achievements were obtained during the **entire period** of the project:

- Parameters were determined to allow a definition of requirements of OVs for selected cancer models. Thus, the Consortium decided to select candidate oncolytic viruses on the basis of distinct tumour-specific phenotypes (compared to normal cells) in: i) oncolytic capacity (tumour specific virus DNA replication, formation of progeny and viral propagation), ii) infectivity (cell entry), iii) spreading (cell-to-cell transfer), iv) tumour cell restricted FCU1 expression upon infection of cancer cells compared to normal tissue.

- Common strategies and methodologies were defined and adapted for oncolytic viruses in the THERADPOX project. Adaptation and harmonisation of strategies was attempted with the goal to be able to compare results among partners. The establishment and harmonisation of common strategies concerned specifically: vector engineering, production and purification of oncolytic Pox and Ad, reporter genes, quality controls, standardisation of therapeutic potency and tumour selectivity, methods for tumour cell infection *in vitro*, common *in vitro* evaluation of the functionality of FCU1, tumour models, standardised analysis of data (all partners). No major alternative strategies had to be implemented.

- Towards enhanced tumour infectivity and enhanced tumour selective replication concerning oncolytic adenoviruses the efforts were divided into two parts: liver de-targeting and tumour targeting (ICO, UH.RDD, USTAN).
Various novel and optimized oncolytic adenoviruses were engineered (chimeric viral capsids, targeting transcriptional de-regulated pathways in tumours (E2F most tumours, Wnt colon cancer)) which show a significantly improved tumour to liver ratio *in vitro* in tumour cells and *in vivo*. Furthermore these viruses have been engineered towards increased replication in tumour cells.

- During the 3rd period of the THERADPOX project, double deleted oncolytic *Vaccinia* viruses were shown to display increased tumour selectivity with respect to tumour selective replication and propagation depending on the specific gene deletions inside the *Vaccinia* virus genome.

- Concerning oncolytic Pox viruses the selective replication of *Myxoma* virus (MV) in tumour cells and their lack of replication in normal cells was confirmed and further studied using a multiple attenuated and deleted vaccinal strain, which proved to be our best promising candidate for an oncolytic MV, setting the basis for *in vivo* studies, which were started in September 2008 (INRA).

- Oncolytic *Vaccinia* viruses harbouring double gene deletions based on the parent virus VVTK have been engineered to increase the selectivity for and constructed for increasing the selectivity of tumour cells and the spreading ability inside the tumour (PEI). Thereafter, they have been tested *in vitro* to assess the functionality of gene deletions (PEI) and *in vivo* to evaluate in mice its efficiency in several tumour models thanks to a collaborative effort between TRANSGENE, ONCOTEST and PEI. To enhance the tumour specificity and to decrease the toxicity of thymidine kinase –deleted onvolytic *Vaccinia* virus vectors (VVTK-), deletion of other genes were combined with the tk deletion. In special, the impact of double gene deletion on oncolytic activity and safety of the different viruses were comparably tested whereby some of the double deleted viruses showed preferable oncolytic activity and tumour selectivity. The double deleted vectors demonstrated equivalent therapeutic activity compared to single deleted vectors. In addition, significant decrease of toxicity was observed with some of the double deleted vectors. Arming of double deleted oncolytic *Vaccinia* viruses for some viruses also enhanced the oncolytic activity without increasing toxicity (PEI, TRANSGENE).
This beneficial effect could be shown for several colorectal tumours derived from cancer cell lines or in one case from a patient derived colorectal tumour in mouse models (TRANSGENE, ONCOTEST, PEI).

- Towards increased spreading of oncolytic viruses through the tumour tissue an oncolytic adenovirus carrying a fusogenic protein has shown increased spreading through tumour cells in culture (ICO). Furthermore, oncolytic adenoviruses targeting stromal barriers in tumour tissue have been engineered (USTAN, UH.RDD). Adenoviruses expressing enzymes digesting stroma are being jointly developed by USTAN and ICO, thanks to a three month visit to USTAN by a member of the ICO group.

The results with Poxviruses show that these viruses have excellent spreading properties. In the case of *Myxoma virus*, the multiple deleted strain virus was found to spread so well that it was not deemed necessary to engineer complicated mutants to improve spread; the further development of *Myxoma* as an oncolytic agent is instead subordinate to a greater understanding of the tumour selectivity of the virus, as described in the WP on replication (WP03.1).
The results with *Vaccinia virus* show that a deep understanding of virus biology can lead to rapid progress in the development of optimised oncolytic viral vectors. Furthermore, the *Vaccinia* groups have taken the HA mutant through multiple further steps of virus engineering, including arming with FCU1, and then tested it in vitro and in vivo, all in the context of the THERADPOX consortium. The resulting virus is a serious candidate for further clinical development and represents one of the major achievements of the THERADPOX program.

- Towards an improved therapeutic efficacy of OVs oncolytic adenoviruses have been "armed" with a gene coding for a non-toxic pro-drug converting enzyme, FCU1 (ICO, USTAN, UH.RDD). Several different approaches were used to express FCU1, including replacement of an E3 gene, fusion to pIX with a picornaviral 2A sequence, and insertion of an additional splice unit in the major late transcript. FCU1 gene has also been inserted in a wild type TK- *Myxoma* virus strain (INRA). FCU1 gene has also been inserted into the TK gene of several MV strain (INRA), and its functionality *in vitro* was confirmed (TRANSGENE). The construction of Ad5/3-D24-FCU1 was achieved, the structure of the virus confirmed and the expression of FCU1 verified (UH.RDD).

- Towards an enhanced safety and controlled release of OVs, follow-up studies on earlier developed formulations continued and new injectable formulations were developed for easier handling & storage and to study different virus concentrations in silica formulations (DELSITECH). More OVs of UH.RDD were encapsulated in different injectable silica formulations as well as silica implants and silica was observed to have an effect on the biodistribution of the viruses. The continuation of the follow-up study showed that there are several silica formulations where the infectivity of viruses is well preserved (at 4°C and room temperature more than 1 year). Injectable silica formulations have further been developed for easier handling in use and for simpler storage and the results showed very stable preservation of virus infectivity at least for 3 months. *Myxoma viruses* (MV) from INRA were encapsulated in silica matrix and inoculated to rabbits. The results show that MV is gradually released from the silica, leading to sero-conversion of all animals, and histological features consistent with what is usually observed with the strain use. The *in vivo* results showed also that the infectivity of the *Myxoma viruses* was preserved in silica at least for 13 months.

- Towards the *in vitro* testing of selected OVs adenovirus permissive ONCOTEST cell lines established from patient derived xenografts were identified (ONCOTEST). A protocol for *in vivo* selection of permissive xenografts has been developed jointly by ONCOTEST and USTAN, with animal injections performed at ONCOTEST and viral quantisation at USTAN.

- The efficacy of selected Ovs for tumour killing *in vivo* was evaluated by systemic administration into nude mice bearing patient derived human tumour xenografts bearing nude mice. Ovs were identified that show a strong anti-tumoural effect alone and in combination with the pro-drug 5FC and a very low toxicity indicated by an acceptable body weight loss of the treated mice.

- Selected oncolytic *Vaccinia virus* vectors and oncolytic Ad vectors were evaluated in patient derived colon cancer xenografts *in* vivo (ONCOTEST). Based on the results of the *Vaccinia virus* experiments, double deleted oncoloytic VV are promising candidates to be further evaluated and to be proposed for clinical development

- A software application, developed to allow fast sharp and standardized analysis of data, has been implemented. Prior to making the software application available to the project partners, rigorous tests were made to validate the following modules: Imaging, Resutls, Bio Bank, Autopsy, Audit Trail, Project & Tasks Planning and the Searching function. The software was used by ONCOTEST within THERADPOX project's framework in order to conduct few *in vivo* experiments. This latter has proven to be an efficient and complete tool to fulfil THERADPOX project requirements.

- The website www.theradpox.org allowed the dissemination of the results to scientific, clinical and citizen communities (NEWLAB).

1.3. Conclusion:

Various model oncolytic Pox and adenoviruses have been generated as proof of concept towards the development of oncolytic vectors with improved therapeutic efficacy.

The THERADPOX project has through translational research generated an armamentarium of different oncolytic Pox – and adenoviruses and different tools to enhance their tumour specificity. The translational research in this project has demonstrated proof of concept for oncolytic vectors with increased tumour-selectivity of infection, replication and viral spread through the tumour tissue.

The preclinical data obtained throughout this project have set the stage for selected oncolytic virus candidates towards entering clinical development in patients with cancers refractory to current treatment modalities. The results of the THERADPOX project facilitate a clinical translation of oncolytic adenoviruses and poxviruses for a variety of different cancer types.

The project has in the third reporting period respected the timelines and no major deviations are reported.
The objectives were reached through a strong consortium, involving academic partners, SMEs and industrial partner.
Furthermore, several inter-partner collaborations have been developed which were not initially planned:

- DELSITECH and UH.RDD UH.RDD has evaluated and confirmed *in vitro* and *in vivo* the functionality of the silica gel-based viral formulations developed by DELSITECH (see WP06).
- INRA and DELSITECH
  - INRA has evaluated *in vitro* and *in vivo* the functionality of the silica gel-based viral formulations (*Myxoma virus*) developed by DELSITECH (see WP06).
- PEI and TRANSGENE (WP03, 04, 05,07)
  - A variety of double deleted oncolytic *Vaccinia* vectors have been generated at TRANSGENE and PEI.
  - In order to expedite the characterization of these vectors, PEI concentrated its efforts on the *in vitro* analysis while TRANSGENE pursued the *in vivo* analysis of these vectors.
- INRA and TRANSGENE (WP03)
  - TRANSGENE collaborated with INRA in comparing various *Myxoma* vectors to oncolytic and non-replicative MVA with respect to the functionality of *Myxoma* vectors *in vitro* as well as the functionality of FCU1.

1.4. Achievements of the THERADPOX project to the state-of-the-art

The arsenal of oncolytic viruses that are under development is ever expanding, as are the creative strategies for achieving tumour-selective replication, tumour cell killing and viral spread towards increased therapeutic efficacy. Owing to the complexities of tumour biology and the heterogeneity of human tissues, it seems unlikely that one kind of virus or a single targeting strategy will be sufficient to treat all cancers.

Although the number of different types of oncolytic viruses that have been tested in preclinical trials is increasing, only a few have made the transition into the clinic. Thus, our clinical experience is limited — there is still an unmet need to study these new therapeutics and developing ways to optimize their efficacy.

The THERADPOX project has optimised the therapeutic class of oncolytic viruses combining targeted and armed approaches for treating cancer. Our preclinical results show that products from this therapeutic class can systemically target cancers in a highly selective and potent fashion using a multi-pronged mechanism of action.

The preclinical data obtained during the project (ICO, UH.RDD, ONCOTEST, TRANSGENE) have set the stage for selected oncolytic virus candidates towards clinical evaluation of safety and efficacy in patients with tumours refractory to current modalities. These results thus facilitate clinical translation of oncolytic adenoviruses and oncolytic *Vaccinia* viruses for the treatment of a variety of cancers, including colorectal, ovarian and pancreatic tumours, as well as renal metastatic breast and gastric cancer. In fact through this project, several oncolytic adenovirus and pox Oncolytic vectors have emerged as very promising candidates to be taken into clinical development.

The THERADPOX project has confirmed Virotherapy as a new paradigm for potent, safe and practical therapeutics for cancer.

1.5. Impact of the THERADPOX project

The THERADPOX project has generated advanced knowledge specifically on the potential of oncolytic *Myxoma virus*, *Vaccinia virus* and adenovirus for tumour eradication and on the therapeutic potential of engineering viruses for optimising eradication of tumours while applying non-toxic compounds.

Apart from engineering and testing OVs, the THERADPOX project has optimised the systemic and intra-tumoural delivery of OVs. In addition, the THERADPPOX project has developed a promising mode of delivery: controlled release using biodegradable sol-gel silica matrix.

Furthermore, a high added value software application has been developed in the project to standardise and accelerate the analysis of numerous and critical data generated during the project.

The THERADPOX project also included a strong involvement of SMEs: Out of 10 partners, 4 SMEs were involved in the project with a significant amount of the total budget

Due to the advanced knowledge obtained in this project it can be proposed to invite interested stakeholders to participate in a Workshop on Oncolytic Virotherapy. This workshop should be hold under the auspices of the European Forum on Good Clinical Practice (EFGCP) and EORTC or through the NoE CLINIGENE, in which TRANSGENE and PEI are partners. Indeed, the objectives would include bringing together stakeholders (patients, caretakers, investigators and other stakeholders) to discuss practical implications/considerations on the conduct of clinical trials with such oncolytic virotherapies as well as to generate a concept paper providing guidance for future sponsors and participants of such clinical trials

The THERADPOX project provided a significant opportunity to increase the competitiveness of Europe in Cancer Research, specifically that of virotherapy. Indeed, the THERADPOX project has developed novel and optimised treatments for a variety of cancers based on original, safe and efficient oncolytic vectors (OVs) (from 3 different viral platforms: VV, MV and Ad) combined with non-toxic chemotherapy. Their spectrum of action could be easily extended since the THERADPOX project included an establishment of standardised protocols and the creation of a software application allowing standardised analysis of data thus accelerating the overall development and production of OVs.