SILVER FINAL Publishable Summary

Small-molecule **I**nhibitor **L**eads **V**ersus **E**merging and neglected **R**NA viruses (**SILVER**)

Project no 260644

Executive summary

The success of vaccines in eradicating smallpox and distemper and reducing many other human and animal virus diseases to very low levels has been a major scientific achievement. However, Acquired Immunodeficiency Disease (AIDS), first officially recognised in 1981, was caused by a retrovirus (HIV) which compromised the immune system of infected hosts. Consequently, vaccines were unlikely to be effective against this disease. This prompted a major search for drugs that could inhibit the replication of HIV and other RNA viruses, including hepatitis C virus and influenza virus. Thus, whilst AIDS is still a major threat to humans in developing countries with poor medical health infrastructures, antiviral therapeutic agents are now significantly extending life expectancy of infected humans in developed countries. However, improving living standards, increasing human mobility, commercial transportation, urbanisation, population density, climate change and other factors have inadvertently created environments perfectly adapted for exploitation by emerging or re-emerging RNA virus pathogens, for which there are currently no vaccines or drugs.

The SILVER project was conceived in response to an EU 7th Framework Call under the theme Health (http://ec.europa.eu/research/health), to develop a drug discovery programme to inhibit RNA viruses that fall into two classes; priority viruses (Flaviviridae, Picornaviridae and Paramyxoviridae) and other neglected and/or emerging viruses which include coronaviruses, alphaviruses, noroviruses, bunyaviruses, lyssaviruses and arenaviruses. A consortium of scientists was assembled with expertise in drug discovery, crystallography, biochemistry, medicinal chemistry, virology, bioinformatics and structure resolution. A pipeline strategy was developed for discovery and problem resolution. It comprised six work packages: (i) cell-culture based screening of compound libraries and mechanism of action studies, (ii) structure-function analysis of viral enzymes in replication complexes, (iii) structure and fragment-based drug design and hit-to-lead chemical modification, (iv) proof of concept (PoC) analysis incorporating toxicity studies and in vivo models, (v) route to market and licencing to pharma (vi) project management. An outbreak pipeline and contingency funds were included in preparedness for emerging viruses. This proved fortuitous as MERS coronavirus, chikungunya and ebola virus all emerged during the SILVER project.

Eight virus families were studied. From > 700,000 cell-based assays, >700 promising hits were identified and chemically modified, 20 of which were tested for (PoC). Target-based discovery led to the design of 186 chemical candidates one of which was tested in vivo. Of 37,000 compounds screened against purified enzymes one showed inhibitory activity in vivo and from hit explosion of chemically modified hits, 2 products are under analysis for PoC. One licence agreement for an enterovirus inhibitor was signed with Novartis for clinical analysis and two more inhibitors are being considered for licencing. Seven patents have already been secured and up to 20 more may follow. Many novel assay systems and processes have been developed and published and, at the time of reporting, 164 peer-reviewed publications are already in press and 15 PhD theses have been completed or are in the process of completion. All partners and associated scientists have participated at international conferences and many have provided advice to health agencies. In summary, SILVER has been a resounding success with the prospect of benefits to mankind in terms of disease control, new therapeutic drugs, new scientific knowledge, education of scientists, quality of life, social impact and cost efficiency in public health.

Summary description of the project context and the main objectives.

SILVER was conceived in response to an EU 7th Framework Call under the theme "Health" (http://ec.europa.eu/research/health). The context was to develop new strategies for health control of emerging and neglected RNA viral diseases, not including HIV, influenza or hepatitis C virus which have been covered under other EU Calls. SILVER is a drug discovery-based research programme to identify and develop, to proof of concept, potential inhibitors of RNA viruses that fall into two classes; the priority viruses (Flaviviridae, Picornaviridae and Paramyxoviridae) and neglected and/or emerging viruses which include, coronaviruses, alphaviruses, noroviruses, bunyaviruses, lyssaviruses and arenaviruses. Based on the experience of proposed partners the likelihood of an unanticipated human pathogenic RNA virus emerging during the 48 month project was high and therefore a contingency fund was built into the project proposal to cover for this eventuality. This proved to be a fortuitous strategy with the totally unexpected emergence of the MERS CoV and unanticipated discoveries of rabies virus inhibitors that needed to be further optimised and tested in vivo. The final objective of SILVER, ie to achieve proof of concept, would lead to patenting the most promising viral inhibitors with a view to licensing the products to pharmaceutical companies who would conduct pre-clinical and clinical studies, necessary to take the products to market.

Concerning objectives, it was agreed that the consortium needed experts in in vitro (medium and high throughput –HTP-) screening and in vivo viral inhibitor assessment, mechanism of action studies, development of viral replicative enzyme assays, crystallography, virtual screening, macromolecular crystal structure determination, molecule/inhibitor interactions at the structural level, viral replication complexes, medicinal chemistry, proof of concept procedures, bioinformatics, IPR, ethical procedures and web-based systems. This base of expertise was established by recruiting European and Asian experts with the main objectives defined. SILVER comprises four scientific Work Packages (WP). A pipeline strategy was implemented to ensure continuity and to facilitate flexibility and the introduction of molecules and compounds for inhibitor studies at all stages of the project. An outbreak pipeline was also activated in response to the newly emerging MERS coronavirus in Saudi Arabia. WP5 was dedicated to IPR issues, dissemination, delivery of products to pharmaceutical industries, ethical, safety and security issues and WP6 was dedicated to management and coordination of the project.

WP1 objectives focused on coordination of laboratories specializing in the RNA viruses defined above to screen potential inhibitors in the form of compound libraries or molecules. The main activities of WP1 throughout the 4 years were: cell-culture based screening (including systematic organisation of these activities in partner laboratories), and mechanism of action (MoA) studies.

WP2 objectives focused on development and determination of crystal structures and drug discovery using replication complexes based on a wide variety of viral target enzymes and RNA or other ligands previously identified as inhibitors.

WP3 developed a rational approach for screening chemical libraries against recombinant macromolecular targets such as viral enzymes. Alternatively, their crystal structures were used to design inhibitors. Three-dimensional structures of targets were resolved and used for virtual screening of chemical libraries. All chemical optimizations of hit compounds, for both WP1 and WP3, were also performed within WP3 and collaboration with WP2 focused on

determination of crystal structures of complexes between viral target enzymes and RNA or other ligands.

WP4 partners received the most active antiviral compounds identified in WP1-3 for ADME/Tox and PK studies as well as evaluation of efficacy in small animal models. In many cases, novel animal models had to be developed to facilitate the in vivo studies.

WP5 Exploitation and route to market (IPR manager). Consortium agreement amendments, monitoring exchanges of molecules and compounds via Wiki. Outreach via congresses attended, links with chemical and pharmaceutical industries, invitations for industrialists to attend Reporting Meetings. Review committee for IPR.

WP6 Organization of Reporting, financial allocations, partner support, preparation of minutes, organisation of SC meetings, management of contingency fund, liaising with Project Officer, contractual/financial management, preparation of Periodic and Final Report.

Description of the main S & T results/foregrounds

Background to emerging human pathogenic viruses

Human virus diseases, such as smallpox, rabies, influenza and dengue fever, have been described in ancient records dating back thousands of years. However, the scientific recognition of the existence of viruses as aetiological agents of disease and their isolation and characterisation essentially commenced during the late 19th and early period of the 20th century. This followed the demonstration of filterable transmissible agents smaller than bacteria. Earlier, in 1848, when yellow fever was still a mysterious disease, Josiah Nott, a physician in Mobile, Alabama noted that places not visited by steamboats, on the Mississippi River, had been uniformly exempt from the disease yellow fever. Unwittingly, Dr Nott had described the first evidence for the introduction and appearance, ie. the "emergence", of a virus disease in a new environment as the result of anthropology. Expressing this in a modern context, anthropology is the major driving force for emergence of pathogenic human viruses.

Subsequently, from about 1930 onwards, many new human, animal and plant pathogenic and even non-pathogenic viruses were isolated and characterised. Indeed by the 1960s, the pre-molecular biology era, many virologists were beginning to believe that most pathogenic viruses had been recognised and consequently there was little more to be discovered. Not surprisingly, nature still had a few tricks up its sleeve!

During the 20th century, despite two World Wars, the Spanish flu pandemic and the Great Economic Depression, population densities, human mobility, urbanisation, land reclamation, deforestation, commercial transportation, scientific methodologies, viral vaccines and many other anthropologically related activities (ARA), were all increasing at an accelerating rate. These and many other ARA were providing the opportunity for RNA viruses to exploit the consequences of anthropology. Thus, by the mid-20th century, the era of emerging viruses was truly born.

The list of emerging or re-emerging human pathogenic viruses is growing rapidly but those that originally attracted the most attention and thus the highest levels of funding for research, into virus disease include HIV, influenza and the hepatitis viruses. Subsequently, enteroviruses, hantaviruses, filoviruses, flaviviruses, alphaviruses, paramyxoviruses, noroviruses, lyssaviruses, coronaviruses and arenaviruses have all gained prominence as emerging viruses. Doubtless, many others are waiting for their turn. Importantly, virtually all of these emerging pathogens are RNA viruses. This is readily explained because RNA viruses that do not possess proof-reading activity have relatively high mutation rates which, during virus replication, result in the generation of huge numbers of genetic variants, often described as quasispecies populations. Thus, RNA viruses have evolved a highly efficient molecular mechanism for adaptation to changing habitats and environmental conditions.
Acquired Immunodeficiency Disease (AIDS), first officially recognised in 1981, is caused by a retrovirus (human immune deficiency virus - HIV) which compromises the immune system of infected hosts. As HIV dispersed across the globe, killing virtually every victim in its path, it became apparent that a major international research effort was required to control this disease. Moreover, early phylogenetic studies demonstrated that the impact of anthropology was greater than anyone could have imagined. It soon became clear that simian species were a reservoir for a variety of closely related simian immunodeficiency viruses (SIV) that were also able to exploit ARA. In other words, the number of potential different but related HIVs could be limitless. Consequently, as the search for control methods evolved it became clear that it was going to be a long drawn out process to develop vaccines that would be effective against these viruses and thus far, this has proved to be the case. However, this fact prompted a major search for drugs that could inhibit the replication of HIV and thus provide an effective alternative to vaccines with which to treat and ultimately control AIDS. The result is that there are now many drugs available with which to treat AIDS patients thereby extending

their life expectancy although the drugs do not actually eradicate the virus from the infected patient.

Armed with the knowledge gained from these successes, it became clear that antiviral drugs do have a future in human and animal virus disease control.

SILVER was conceived in response to an EU 7th Framework Call under the theme Health (http://ec.europa.eu/research/health), to develop a drug discovery programme dedicated to the identification of inhibitors of emerging and neglected RNA viruses that fall into two classes; priority viruses (Flaviviridae, Picornaviridae and Paramyxoviridae) and other relatively neglected viruses which include coronaviruses, alphaviruses, noroviruses, bunyaviruses, lyssaviruses and arenaviruses. Why do we use the term "neglected viruses"? This term is used to reflect the fact that although many of these newly emerging RNA viruses are important in terms of human morbidity and mortality, when compared with other disease agents and other factors, including "numbers of road deaths" the actual numbers of deaths are relatively small (Annex Figure 1) and therefore are not financially attractive to the major pharmaceutical companies.

Figure 1

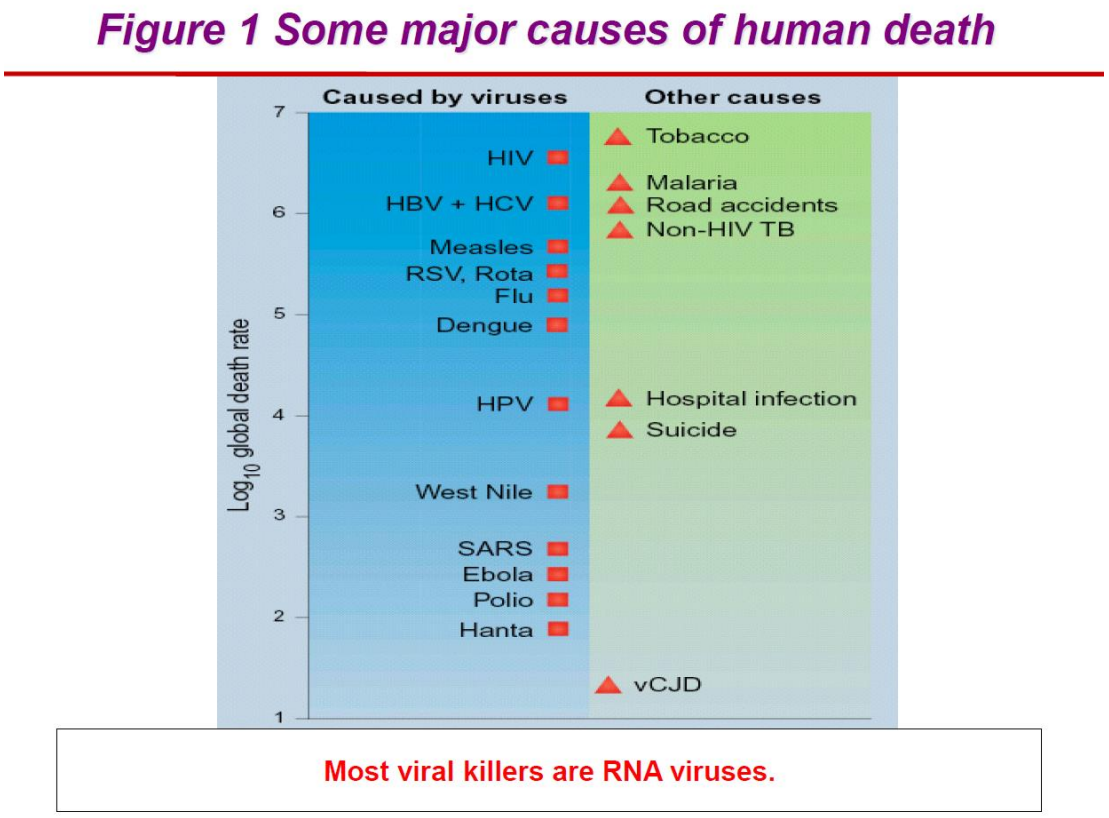


Figure 1 presents a comparison of relative human death rates due to relevant emerging and neglected viruses and compares these rates with other causes of death in humans. Note that virtually all these viruses are RNA viruses.

It has to be emphasised that the SILVER Steering Committee recognised from the instigation of the project that the discovery programme could not realistically proceed beyond the stage of Proof of Concept (PoC), ie the demonstration that identified viral inhibitors were effective in in vitro tests (ADME tox and PK studies), did not induce genetic resistance readily and were effective in in vivo laboratory models. Thus, any effective, non-toxic inhibitors discovered in SILVER which satisfied the criteria for PoC, would have to be patented and

then licenced to pharmaceutical or other relevant companies with adequate funds to take them through the clinical trials and then to market.

The SILVER project: Workflow and Pipeline strategies

In response to the EU Health Call, a consortium of scientists was assembled with expertise in drug discovery via cell culture-based screening and mechanism of action (MoA) studies, animal model test systems for viruses, analysis of ADME-tox and PK properties, in vivo efficacy studies of promising compounds, enzymology, crystallography, biochemistry, medicinal chemistry, virology and bioinformatics. Two SMEs - Global Phasing, Cambridge, UK (which specialises in methods of macromolecular X-ray crystallography and integrated software for drug discovery and structural biology research, and Riboxx, Dresden ("RNA in a box" – which produced and supplied large quantities of high concentration synthetic RNA), were also partners within the SILVER consortium.

A workflow programme based around work packages (Figure 2) and a pipeline strategy (Figure 3) for discovery and problem resolution were developed to ensure continuity and close collaboration between SILVER partners. The programme comprised six work packages: (i) cell-culture based screening of compound libraries and mechanism of action studies, (ii) structure-function analysis of viral enzymes in replication complexes, (iii) structure and fragment-based drug design and hit-to-lead chemical modification, (iv) proof of concept (PoC) analysis incorporating toxicity studies and in vivo models, (v) route to market and licencing to pharma (vi) project management and coordination. An outbreak pipeline and appropriate contingency funds were included in preparedness for new emerging viruses.

Workgroups for Advancement, Validation and Exchanges (WAVES) were also built into the work programme at the sub-work package level. Each WAVE comprised a Leader and small multi-disciplinary groups of scientists, dedicated to solving problems, circulating up to date information, developing protocols and evaluating progress towards the milestones.

Figure 2

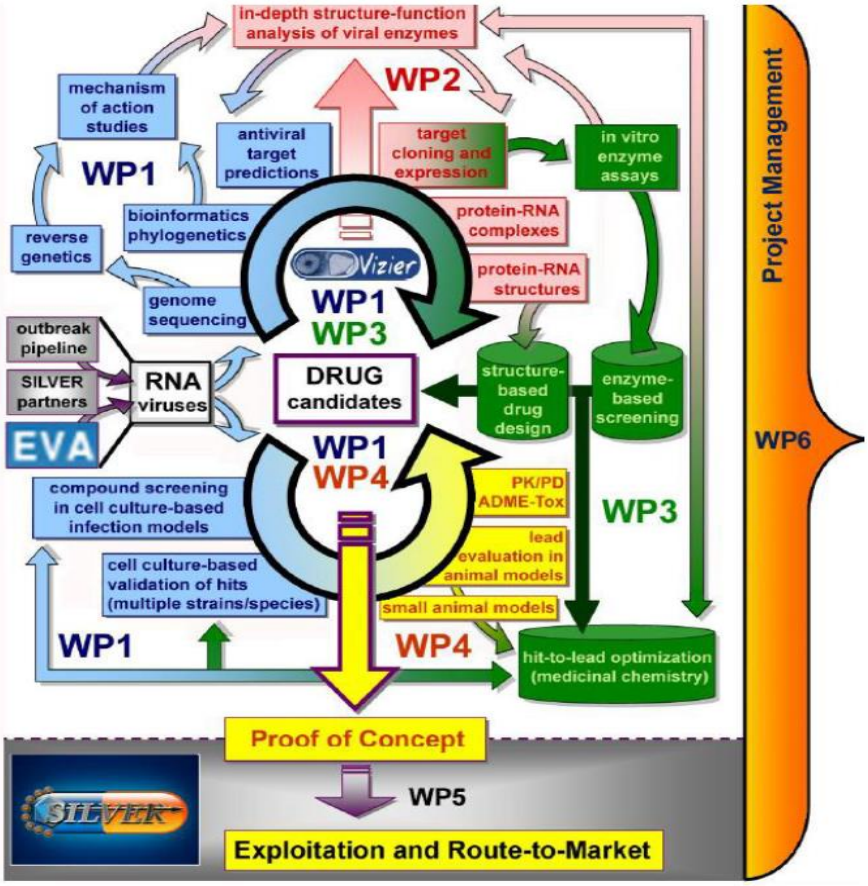
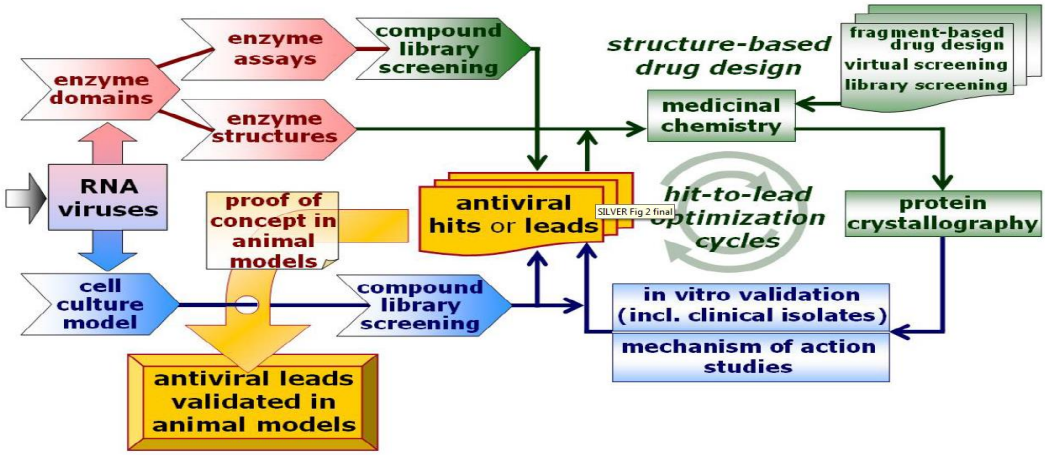


Figure 2 represents the strategy for workflow as described in the text of the Final Report. The colours of the individual Work Packages emphasise how each can dovetail into the others thus improving collaboration and interaction between partners in individual groups.

Figure 3 Pipeline strategy



Viruses introduced at all levels of existing knowledge.
Drug targets - infectious virus, structurally characterised enzymes, replication complexes, other proteins.
Drug leads identified by screening compound libraries in cell culture, and *in vitro* viral enzyme assays.
Inhibitors of replication, derived using structural knowledge of proteins & structure-based drug design.

Figure 3 represents the pipeline strategy adopted to ensure continuity and entry of compounds/molecules for inhibitor studies at any point of progress within the SILVER projects. This pipeline strategy also proved to be ideal as an Outbreak Pipeline for research funded by the Contingency Fund when new viruses emerged.

Outline of the results:
Specific details (Figures/Tables) of all results reported below can be found in the SILVER Period Reports for years 1 to 4.

WP1

Work package 1 entailed the coordination of laboratories specializing in a wide range of emerging and/or neglected RNA viruses (9 important families with positive- or negative-stranded RNA genomes) to initiate and implement screening of potential inhibitory compounds in the form of either compound libraries or molecules previously identified as (potential) inhibitors. The main activities of WP1 throughout the 4½ years of SILVER were: cell-culture based screening (including the systematic organisation of these activities in different partner laboratories) and mechanism of action (MoA) studies.

The screening campaign (Task 1.1) started with the gathering of appropriate reagents and tools, plus hiring and training of personnel. The extensive cell culture-based screening campaign of compound libraries was led by P3-KULeuven in collaboration with other partners from inside the consortium. It also involved several logistic challenges that were handled successfully (e.g. transport of ready-to-go compound library plates to partner laboratories P6-IP and P1a-AMU). During SILVER year 3, the chemical library screening on viruses from eight RNA virus families was completed as planned (priority group A: enteroviruses, flaviviruses, and paramyxoviruses; priority group B: noroviruses, alphaviruses, coronaviruses, rhabdoviruses, and bunyaviruses). The phenotypic screening for new antivirals entailed (i) optimization and validation of the assays, (ii) screening of the compound libraries, (iii) selection and resupply of the hits by the library owners and confirmation of the hits, (iv) selection, re-supply and testing of commercial analogues of the confirmed hits. Subsequently, in-house synthesis and chemical validation of selected hits was performed under WP3. All of the RNA virus families proposed in the original outline of the project were included, and no major setbacks were experienced.

From the library screening programme multiple hits were discovered for each of the selected viruses in the different families. In total > 500 hits and analogues of hits were re-ordered for confirmation. From these ~15 chemical series were selected for further follow-up either by selection of more analogues or by hit-optimization in chemistry programmes. Most of these hit optimization programmes are still ongoing, but 3 chemical series have already progressed to in vivo testing (2 series of rhabdovirus inhibitors and 1 series of alphavirus inhibitors). In addition to the library screening, different partners discovered the antiviral activity of molecules from alternative sources (e.g., FDA approved drugs, collaboration with chemists, derived by structure-based drug design). Of these, 6 selected compounds progressed to in vivo efficacy studies (enteroviruses, flaviviruses, paramyxoviruses, noroviruses, alphaviruses and rhabdoviruses). The assays developed for Task 1.1 (and 1.3) were also used to process a number of compound series provided by WP3 partners for enteroviruses and flaviviruses, which derived from structure-based drug design of library screening using enzymatic assays (Task 1.2).

Towards the end of year 2 (Autumn 2012), SILVER's outbreak pipeline (Task 1.6; coordinated by P4-LUMC) was activated for the first time to address the potential threat posed by the emerging MERS-coronavirus (MERS-CoV), a previously unknown zoonotic coronavirus considered at the time potentially to be a threat to public health in the Middle East and Europe, with the worst case scenario of mimicking the SARS coronavirus and dispersing worldwide. Supported by SILVER contingency funds, nine SILVER partners with different backgrounds and expertise (virology, bioinformatics, enzymology, structural biology, and medicinal chemistry) collaborated closely on the characterization of the new

virus, the development of a MERS-CoV toolbox, and the identification and development of inhibitory compounds and strategies. Major achievements included;

(i) detailed information on the genome organization and molecular biology of MERS-CoV;
(ii) the generation and analysis of several crystal structures of MERS-CoV enzymes and biochemical assays to test their activity/inhibition;
(iii) cell culture-based screening assays to discover MERS-CoV inhibitors;
(iv) development of a small-animal model (rabbits) for in vivo MERS-CoV infection studies;
(v) discovery of a variety of MERS-CoV inhibitors obtained through either cell-based screening or structure-based drug design, some of which displayed potential as pan-coronavirus inhibitors;
(vi) seven high-impact publications on MERS-CoV (thus far);
(vii) a variety of ongoing projects and starting points for future collaborative antiviral research on coronaviruses in general, and MERS-CoV in particular, for which additional funding remains to be secured.

Although, not formally recognized as part of the outbreak pipeline task, very similar activities were launched to target the emerging chikungunya virus (CHIKV), in particular after it crossed the Atlantic Ocean to initiate a major outbreak in the Carribean, Central America and subsequently North and South America. At this time, CHIKV also dispersed from south-east Asia across the southern Pacific Ocean and within months the virus was present throughout the entire region of Polynesia, an area larger than the entire European land mass. A large number of partners collaborated to discover and advance a variety of antiviral hits for this virus.

The MoA studies (Task 1.3) also encompassed the development of a variety of experimental systems (Task 1.4) which included reverse genetics platforms and biosafe screening systems for several virus groups. These studies were supported by in-depth bioinformatics analyses (Task 1.5; P4-LUMC). These virus-specific toolboxes were subsequently used for both hit validation and MoA studies, which revealed information for inhibitors of the replication of five different groups of RNA viruses, including two of the project's priority viruses, summarized below:

<u>Enteroviruses:</u>
• KRICT compound series and Fluoxetine, which target the viral 2C protein.
• TZ, OSW-1, TTP-8307, which interfere with the enterovirus host factor OSPB that mediates lipid fluxes important for replication organelle formation.
• TP219 targets enterovirus capsid protein VP1 by scavenging glutathione which is essential for enterovirus encapsidation.
• GPC-N114: Interferes with RNA replication by occupying the RNA template channel in the enterovirus RdRp.

<u>Flaviviruses:</u>
• ST-148: Enhances dengue virus capsid protein self-interaction, presumably inducing structural rigidity and/or steric hindrance during nucleocapsid assembly and disassembly.

<u>Alphaviruses:</u>
• Favipiravir (T-705) inhibits the chikungunya virus RdRp through a mechanism other than lethal mutagenesis; resistant mutants have mutations in nsP4 and other nsPs.
• The MADTP and CHVB series inhibit the MTase and GTase activity of alphavirus nsP1. Target and activity confirmed in biochemical assays and cell culture, including reverse genetics.

Coronaviruses:
• Based on the crystal structure of the BatCoV-HKU4 main protease, a number of inhibitors (Michael acceptors, alpha-ketoamides, and aldehydes) have been synthesized and were found to be active against MERS-CoV in cell culture.

Rhabdoviruses:
• RHABDO 2011-Rabies-01 compounds (CIM114116 and CIM114669) inhibit lyssaviruses by targeting the polymerase (L) and phosphoprotein (P) within the viral replication complex.

The bioinformatics component of WP1 (task 1.5; P4-LUMC) supported the antiviral discovery process by advanced virus genomics and contributed to developing a SILVER outbreak pipeline for rapid molecular dissection of newly emerging RNA viruses. The access to the state-of-the-art input on genome sequences and alignments was provided according to (and beyond) the expected deliverables.

In conclusion, SILVER WP1 has been spectacularly successful in generating a large number of hits resulting in several promising leads for many of the viruses covered in this work package. Differences in the hit rate for viruses from different families reflect the relative unpredictability of the methods. Nevertheless, the methods employed represent the most up to date cell-culture based procedures for viral drug discovery. Future discoveries should lead to more precise forms of screening with even higher levels of automation and throughput. Work package 1 completed all its deliverables on time, providing both hits and MoA information for a variety of RNA virus groups. This WP also displayed flexibility and the capacity to deal with unexpected developments which in this case turned out to be the emergence of a unique virus, viz. MERS-coronavirus and the incredibly rapid global dispersal of chikungunya virus throughout the New World, and Polynesia in the Pacific Ocean. Despite the inherently unpredictable nature and timing of these types of outbreaks, and the fact that it cannot be anticipated to which virus family newly emerging pathogens will belong, it can be concluded that SILVER's response was timely and meaningful. Although antiviral drug development in general remains a time-consuming process, high quality hits and leads for the discovery of further antivirals against RNA viruses were obtained, trans-European collaborations were initiated and strengthened, and through this work package SILVER has contributed to creating a better starting position to combat future emerging RNA viruses.

WP2
This work package started with the gathering of appropriate reagents and tools, plus hiring and training of personnel.

During the past three decades, important efforts have been made to develop concepts, technologies and research methods in the sphere of antiviral drug discovery. The success of antiretroviral therapies has spurred great hope that drugs can effectively control a much wider range of antiviral infections. Following the AIDS crisis and first successes in drug development, the RNA virus hepatitis C virus (HCV) has become a target of choice by transposing important knowledge and know-how from the HIV to the HCV field of research.

In both cases, it rapidly became apparent that crystallographic models could play an essential role in the design and refinement of potent drugs by addressing problems relating to drug efficiency and mechanism of drug resistance. However, it also became very clear that a crystallographic model is a static snapshot of one conformation of the target, and that this conformation does not necessarily represent the real-life appropriate target ie the authentic target. Hence, major efforts are being devoted to generating biologically relevant models with which to maximize the likelihood of discovering potent inhibitors and therefore drugs. One important spin-off and benefit of this concept is that by creating and crystallizing authentic complexes, the requirement for extensive in vivo inhibitor studies should ultimately be

reduced, thus potentially fulfilling the principles advocated in the "Three RRRs" (Refinement, Reduction and Replacement).

Replicases encoded by different viruses adopt widely different conformations when binding to their RNA substrates. The severe lack of crystal structure studies of RNA/replicase complexes has lagged behind the ability of virologists to screen identify and characterise drug candidates using state of the art high throughput systems. For example, only very recently a model of a ternary complex comprising nucleotides, RNA, and HCV polymerase was finally structurally characterized following almost two decades of research to reach this point. Clearly, when SILVER was initiated there was a crucial need to resolve this chronic imbalance.

The WP2 aims were to provide the precise structural description of viral targets in complex with RNA and relevant substrates/inhibitors. To achieve this, we exploited the most appropriate and advanced technology available to produce large quantities of high concentrations of biologically relevant RNA substrates (task 2.1) and then delivered them to the relevant target-driven tasks. Selected relevant viral enzymes were crystallized in complex with these RNA substrates and both uninhibited and inhibited complexes were mechanistically characterized (tasks 2.1-2.5, and with WP3). Major efforts were made to focus on the proteins of the three SILVER priority viruses viz Flaviviridae (dengue viruses) (task 2.3), viruses in the family Picornaviridae (task 2.4), and viruses in the family Paramyxoviridae (task 2.5). The Workflow programme and Pipeline Strategy were used as the guide for planning these experiments. Other numerous selected viral enzymes had also been studied at an earlier stage, to advance knowledge and technical know-how towards hit discovery (task 2.6). Defined and relevant RNA substrates were synthesized chemically (P11 and P18) and/or enzymatically (P1b) and optimized in sequence, size and binding affinity. They were produced and purified on a large scale (P1b, 9, 11), and used in co-crystallization experiments (P1b, 12, 13). Crystal structures of the complexes were determined in the presence and absence of inhibitors, in close collaboration with partners in WP3. Differential activities and activity levels between liganded and unliganded enzymes (RNA, activators, inhibitors, co-substrates) were also addressed in order to determine unambiguously the inhibited form/conformation of the viral target and deliver relevant mechanism-of-action data.

The key achievements of this section are summarised below:
- We developed a reliable and high yielding synthetic route towards DNA- and RNA- 5'-capped or ending with triphosphates.
- We developed structural and functional models of dengue virus NS3 and NS5 replication enzymes.
- Numerous original crystal structures of enterovirus proteases and polymerases, were generated for studies with substrates, under several mechanistically relevant conformations
- A variety of crystal structures and crystals of Matrix and L polymerase fragments in complex with RNA capping substrates of clinically relevant mononegavirales (hMPV, rabies virus) were generated and studied
- Several original crystal structures and the mechanisms of action in complex with ligands from emerging and potentially emerging viruses, including coronaviruses (including MERS-CoV), noroviruses, hepatitis E virus, chikungunya virus, arenaviruses and bunyaviruses were resolved.

The studies and achievements emanating from the work of partners in WP2 represent probably the most comprehensive analysis of "authentic" in vitro generated RNA viral replication complexes, over a 4.5 years period, ever described. These successes represent the excellent contributions of SILVER partners in all four work packages but particularly in WP2.

Clearly there is much more work to do but we believe we have opened many doors through which we can look forward to an exciting future using these concepts to expedite the discovery of new RNA viral drugs.

WP3

Within the SILVER project, two alternative but complementary approaches were chosen to discover new antiviral compounds. Work package 1 (WP1) used medium- and high-throughput screening of chemical libraries against viruses in cell culture, without knowing the molecular target of the compounds exhibiting antiviral activity. In contrast, a rational approach was chosen in WP3 in which chemical libraries were screened against recombinant isolated macromolecular targets, often a viral enzyme. Alternatively, the crystal structure of a target was used as the basis for the design of inhibitors. In addition, the three-dimensional structures of the targets were also used for virtual screening of chemical libraries. Each approach to antiviral drug discovery has its advantages and disadvantages. Therefore, in order to maximise the possibilities of success in identifying potential antiviral drugs, a multidisciplinary approach was adopted.

There was also considerable collaboration and interaction between partners in different work packages, in particular WP1 and WP3, because all chemical derivatization of hit compounds was performed within WP3, whether the hit came from WP1 or WP3. There, was also excellent collaboration between partners in WP2 and WP3, which focused on the determination of crystal structures of complexes between viral target enzymes and RNA or other ligands. Finally, the most active antiviral compounds from both WP1 and WP3 were channelled into WP4 for ADME/Tox and PK studies prior to their evaluation for efficacy in small-animal in vivo models.

In this section, the main S & T results obtained within WP3 throughout the duration of the project are highlighted.

<u>New antivirals / new chemical entities</u>

P1b-AMU/AFMB

Over the duration of the SILVER project, P1b discovered two series of compounds (1765 series and 3438 series) with activity against the dengue virus polymerase, and subjected them to hit-to-lead optimization. A focused library was synthesized and used for studies of structure-activity relationships.

P2-UzL and P19-SIMM (with P3-KULeuven and P4-LUMC) used a structure-based approach to develop inhibitors against the 3C protease of enteroviruses and the main protease of coronaviruses. Importantly, more than 30 crystal structures of protease complexes were determined in this sub-project. Three classes of active compounds were developed: Michael acceptors (with SG85 as most potent inhibitor), □-ketoamides (DZL08), and aldehydes. Some of these compounds have shown superb activity against human rhinoviruses, enteroviruses 68 and 71, MERS-coronavirus (EC50 = 200 pM), and SARS-coronavirus. Notably. SG85 was able to abolish the histological markers for pancreatitis caused by Coxsackievirus B4 in a mouse model (P3-KULeuven).

P2-UzL determined the crystal structures of the main proteases of bat coronaviruses HKU4 and HKU8, which belong to the genera of Betacoronavirus (clade c) and Alphacoronavirus, respectively. The goal of these studies was to improve preparedness against future zoonotic transmission of coronaviruses from bats to humans. Some inhibitors previously synthesized by P2-UzL or P19-SIMM proved active against the HKU4 (but not the HKU8) Mpro and new compounds were designed and synthesized on the basis of the HKU4 Mpro structure.

The complex of this enzyme with P2's Michael acceptor compound SG85 was also determined. Proof of concept came with the emergence of MERS-coronavirus (MERS-CoV) in late 2012: SG85 was shown to have excellent antiviral activity against MERS-CoV in Huh7 cells and moderate activity in Vero cells (P4-LUMC).

P10-Oxford determined the crystal structure of the whole enterovirus A71 (EV-A71). Analysis of complexes with four 3-(4-pyridyl)-2-imidazolidinone derivatives (from P17-UIBK) with varying anti-EV-A71 activities pinpointed key structure-activity correlates. On this basis, additional potentially beneficial substitutions were identified and two more derivatives synthesized. Structural analysis and in-vitro assays confirmed the binding modes predicted by modelling and their ability to block viral infection. One of the resulting compounds had an IC50 of 25 pM and showed improved solubility.

P13-CSIC determined a crystal structure of the Coxsackievirus B3 (CVB3) 3D RNA-dependent RNA polymerase in complex with GPC-N114 (from P17-UIBK) as well as that of the encephalomyocarditis virus (EMCV) 3D, both wild-type and a resistance mutant of the enzyme. The same group also determined the structures of the whole capsid of Human Rhinovirus 14 in complex with the canyon binder, LPCRW-0005 (discovered by P3-KULeuven).

P20-KRICT and P3-KULeuven discovered a series of anti-enterovirus compounds that target the 2C NTPase/helicase. Along with two additional series developed by P24-CISTIM and P3-KULeuven directed at the same enzyme, “the compounds were licensed to a major pharmaceutical company”.

P21-NCU optimized a hit series consisting of polycyclic compounds that was originally discovered by P24-CISTIM and P3-KULeuven in a screening campaign against enteroviruses. The most potent inhibitor in this series is now the pentacyclic compound AP-C-003, with EC50 = 2.29 uM, CC50 = 76.4 uM.

P21-NCU pioneered the usage of conjugate libraries to discover new antivirals. They found seven 4-anilinoquinazoline–coumarin conjugates with anti-chikungunya virus activity in the one-digit uM range.

P22-UMIL determined crystal structures of the RNA-dependent RNA polymerase of human norovirus in complexes with either suramin or pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro4',8'-disulfonate (PPNDS), the latter in the presence of RNA (poly(C)-oligo(G) (G12)). Several derivatives and fragments of suramin were synthesized by P21-NCU, with the goal of increasing the membrane permeability of the hydrophilic compound; some of these also had crystal structures of their complexes with the human norovirus RdRp determined.

P22-UMIL identified the anti-helminthic drug Ivermectin as a potent inhibitor of the flavivirus NS3 helicase domain. It also proved a potent yellow fever virus (YFV) replication inhibitor (EC50 values in the sub-nanomolar range). Solubility and toxicity problems were partly overcome through Ivermectin-loaded liposome formulations (external collaboration with Prof. C. Nastruzzi, U. Ferrara, Italy), and tested in cell-based assays (in collaboration with Prof. S. Vasudevan, Singapore). Since 2014, Ivermectin has been in clinical trials in Thailand as a dengue therapeutic (see https://clinicaltrials.gov/ct2/show/NCT02045069); outcomes of the trials will become public in 2017.

P26-FUB developed the first fragment-based broad-spectrum inhibitors of enteroviral proteases, which have potential to overcome the problems of peptidomimetic inhibitors. The compounds showed broad-spectrum activity against a number of enteroviruses (P25-UU). P13-CSIC determined the crystal structure of one compound (NZN) in complex with the 3C protease of enterovirus 93.

During the first years of SILVER, P3-KULeuven together with P6-IP screened compounds from the P1b-AMU/AFMB library for anti-rhabdovirus activity in a cell-based assay. One of the hits was confirmed active with an IC50 of 10 µM and no signs of cell toxicity. P24-CISTIM joined the collaboration and initiated optimization of the series. The hit explosion consisted in the acquisition and testing of 20 commercial compounds in order to learn about a potential early structure-activity relationship (SAR). Once a successful chemical validation of the hit compound was obtained, the series entered hit-to-lead optimization with more than 140 analogues synthesized and evaluated for their capacity to inhibit RABV. A pharmacophoric core was established and the IC50 reached the 70-nM range.

Method development

P1b-AMU/AFMB further developed the method of fragment-screening by a combination of biophysical measurements and X-ray crystallography, using the dengue virus methyltransferase and helicase as targets.

P14-GPhL developed an X-ray crystallography pipeline called "Pipedream" for the large-scale, high-throughput screening of putative target-ligand complexes, particularly well-suited for fragment-based drug discovery. The second major programme developed by P14 as part of SILVER is "Grade", a restraint dictionary generator capable of producing the geometric restraints for arbitrary organic ligand molecules that are indispensable to maintain good ligand geometry while refining target-ligand complexes. P14's entire software suite was made available to the SILVER partners involved in using X-ray crystallography at the privileged "beta release" stage otherwise reserved for P14's commercial sponsors.

P13-CSIC and P2-UzL established the technique of fragment-screening by Saturation-Transfer Difference (STD) NMR spectroscopy in the search for inhibitors of viral proteases, i.e. the 3C proteases of enterovirus B93 and Coxsackievirus B3 as well as the NS2B-NS3 protease of West Nile virus.

P26-FUB developed new methods for fragment ligation, which yielded useful inhibitors for the enterovirus 3C protease and the West Nile virus NS2B-NS3 protease.

P25-UU developed in-vivo protease assays for the enzymes of picornaviruses. P2-UzL used this principle to develop in-vivo assays for the main protease of SARS-coronavirus and MERS-coronavirus.

P21-NCU developed a novel method to generate imidazolidines and pyrrolidines by employing the "A + 2 B" and "A + B + C" synthetic routes. The new tandem reactions use starting materials that are readily available. The reactions generate the products in good to excellent yields, under mild conditions. The entire process is a "single-flask" reaction so that isolation of intermediates is unnecessary. This "green" process is efficient and unprecedented. Overall, we believe WP3 has surpassed all expectations.

WP4

The overall success of SILVER will be judged largely on the basis of the results emanating from work package 4 (WP4) which is devoted to identifying compounds/molecules that satisfy the criteria for achieving Proof of Concept. Consequently, in order to assess candidate viral inhibitors, the research undertaken in WP4 had three primary objectives: (i) the establishment of robust, and in many cases novel, animal model test systems for RNA viruses for which, at the start of the project, there are no satisfactory test systems, (ii) the study of ADME-tox properties of candidate viral inhibitors for in vivo use (iii) in vivo efficacy studies

of compounds with promising in vitro and ADME-tox properties. Each of these objectives is an essential component for the discovery of antiviral drugs, leading to Proof of Concept, patenting candidate inhibitors and ultimately, licencing them with pharmaceutical companies for subsequent assessment in clinical trials (this phase is beyond the scope of SILVER).

Establishment of novel animal model inhibitor test systems for viruses for which, at the start of the project, there were no satisfactory test systems were successfully developed during the first years of SILVER for dengue virus (DENV) and human metapneumovirus.

P5-EMC and P3-KULeuven implemented the AG129 mouse model to study DENV infection. The experiments performed in this mouse model inhibitor test system demonstrated the presence of acceptable viraemic levels, ie with relatively high titres detected in blood and various organs including the spleen, liver, intestine and kidney of DENV-2/NGC, DENV-2/D2Y98P or DENV-2/DKD infected animals. However, the DENV-1 and DENV-3 strains used for these studies did not replicate well. The sylvatic dengue virus type 2 strain (DENV-2/DKD811), which was isolated from a Malaysian patient with dengue haemorrhagic fever was also tested in monkeys by P5-EMC. These experiments indicated that the DENV-2/DKD811 strain replicates better in cynomolgus macaques and virus was detected until day 8 post-infection when administered intravenously.

P5-EMC had previously established a Syrian Golden Hamster model to study human metapneumovirus infection (MacPhail et al., 2004). In the context of SILVER this model was further extended to study virus transmission. Therefore infection/transmission studies for both genotypes of human metapneumovirus (HMPV) in Syrian Golden Hamsters were developed. Both genotypes of HMPV replicated to high titres in the animals, and were shown to transmit the virus through direct contact, ie between animals in close association. For genotype A the studies were extended to demonstrate specifically that transmission between animals in close contact occurred presumably via the generation of infectious aerosols although this need to be confirmed.

Also animal model test systems for other virus infections were developed during the course of the SILVER project. These include rodent models for Enterovirus 71, yellow fever virus and murine norovirus, studied at P3-KULeuven. Additionally, several animal model test systems were explored for suitability to study Middle East Respiratory Syndrome coronavirus (MERS-CoV) infection and inhibition by P5-EMC.

The second objective of WP4 was to study the ADME-Tox profile of novel chemical molecules that had been discovered in other SILVER work packages and which had shown promising in vitro antiviral potency. This task was therefore dedicated to selection of the most potentially suitable inhibitory molecules for subsequent in vivo efficacy testing. This was achieved for novel inhibitors (discovered during the SILVER project) of rabies virus and chikungunya virus.

The rabies series of inhibitors, identified and developed in collaboration between partners P3-KULuven, P6-Pasteur and P24 CISTIM were transferred to WP4 and evaluated in in vitro ADME assays (plasma and mouse liver microsomal stability and plasma protein binding). Four inhibitor compounds that were approved during these in vitro assays, were then tested in mouse PK studies (single dose (i.p.)) to assess their relative levels of brain permeability. It is an absolute requirement for inhibitors of rabies viruses that they must exhibit high brain permeability. This work was outsourced by P24-CISTIM to known CROs. Two of the four compounds that showed acceptable brain permeability were re-synthesized in sufficient amounts to enable further studies. One compound was then explored further in a mouse safety study and did not induce any clinical signs of toxicity. Both compounds were then explored in in vivo inhibitor efficacy studies against rabies virus. The results of these

experiments were promising and further studies and optimisations are currently being undertaken to improve their efficacy as inhibitors of rabies virus in vivo.

The in vitro work of SILVER in WP1 also led to the discovery of new chikungunya virus inhibitors which, interestingly, displayed novel mechanisms of action. For 2 chemical series (the CHVB and MADTP series) ADME-Tox profiling was performed. Based on these results which were favourable, in vivo efficacy studies are now being planned. These studies will be performed in association with a collaborative group of experts and this work will continue following the termination of the SILVER project.

The final objective of WP4 was to study the in vivo efficacy of optimized hits in model systems that had been established previously. This has been achieved successfully with a novel class of enterovirus inhibitors for which we observed 100% efficacy in a mouse coxsackie B4 pancreatitis model test system when a dose of 2 mg/kg was used. As far as we are aware, this is the most active compound class ever studied in this virus/animal model test system. This series is currently being developed further in collaboration with a major pharmaceutical company. During the SILVER in vitro studies (WP1) it was discovered that the inhibitor T-705 (Favipiravir), an approved anti-influenza drug, is also a potent inhibitor of CHIKV infection in vitro. We therefore tested this compound in vivo in WP4 and demonstrated potent activity in the previously developed mouse mortality model test system for chikungunya infection. The inhibitor showed activity both in a disease prevention application as well as in a post-exposure setting. Currently we are investigating if this molecule can also cure chronic CHIKV infection. In a similar way in vitro work in WP1 led to the discovery that T-705 (Favipiravir) inhibits human metapneumovirus infection in vitro. The subsequent in vivo efficacy studies in WP4 showed promising results. In all treated/challenged animals, viral RNA remained detectable in the respiratory tract, however at the highest dose tested a significant decrease in viral RNA titres was observed. Also for 2 anti-rabies virus compounds, discovered in WP1, in vivo efficacy studies were performed. For both compounds a promising trend to improved clinical signs was observed. More research is ongoing to improve the PK of the molecules and to study the effect of these inhibitors on mortality in this model system.

Conclusions

The results presented in WP4 demonstrate the high level of success that has been achieved during the SILVER programme. Novel chemical molecules and compounds have been discovered and taken through the process of optimisation, and assessment in vivo prior to being judged to have reached the level of achieving Proof of Concept. Interestingly, some of these candidate inhibitors display novel mechanisms of action which in itself could lead to future drugs. Additionally, we have demonstrated novel antiviral specificities within the SILVER context, for current approved drugs that are currently being used to treat patients against virus infections not included in the SILVER brief. Several of these drugs showed efficacy in our developed animal model test systems and are potential starting points for novel drugs to prevent or treat emerging and neglected virus infections in humans.

WP5

Access to SILVER Foreground IP was provided for external users both from the academic and industrial community via a unique entry point (the Technology Transfer Platform, TTP). The purpose of the TTP was to promote the work of the partners to the industrial and academic scientific community. The TTP also featured a dedicated website including the description of the SILVER project, promotion activities through presentations, contracting issues, products available and information on SILVER meetings.

In order to increase the visibility of the scientific outputs of the project and thereby to monitor output, criteria were developed to evaluate the progress of the pipeline. The criteria selected comprised the following headings: virus family, the stage of drug development, the

major experiments involved, the results, and relevant questions and methods. Cross collaboration within the consortium to achieve classification of the results was also included.

The work package classification highlights the speciality of the partners, and their contribution to the consortium. The WAVE classification discloses specifically the advances towards emerging viruses or virus families. Thus, this document also monitors the exchanges of molecules and compounds. The rolling basis of this document is totally open and completed by the partners. It is available online in the Wiki part of the consortium secured area. The new task-dedicated projects that arise from the SILVER pipeline are then recorded and assessed by the steering committee for funding from the contingency fund. The results generated that lead to IP are dealt with by the partners, the TTP is consulted only in matters of conflict of interest, or negotiation for further exploitation. These pragmatic procedures ease the progress to market.

During the final year of the project considerable effort was invested in the preparation of patent applications as appropriate. The IPR manager and the Business developer assisted the partners to define their strategy to protect their IP. Impressively, more than twenty compounds are already engaged in patent filing or will be filed during 2015 or 2016 (this includes 8 patents that have already been taken out). The TTP has played a key role through case analysis and advice to define possible means to protect the results generated by the partners. Particular attention was paid to ensure that all partners involved in the development of a drug, were involved in the establishment of the IP.
In terms of IPR, exchanges took place directly with partners. This approach ensured the highest level of confidentiality. In most cases a strategic approach was employed to evaluate the exchanges. In cases when Science was the reason for exchanges, telephone conferences or direct discussions were the means of communication.

Relevant congresses have been attended globally to establish links with the major chemical, pharmaceutical and biotechnology companies: the European antibody congress, BBMRI kick-off meeting, and opportunities for collaboration were sought with major industrial companies such as Novartis (Singapore). The critical pathway of antiviral drug development was disclosed and discussed in depth to determine the role to play by the European consortium with the industrial companies. Indeed, from the first period, the needs and potential collaborations were discussed with the scientific community (public or privately funded institutions; SME's, global emergency network, large industrial companies).
Thus, the double-entry organization that the SILVER consortium developed with the regular work package organization and the flexible WAVE format provided the opportunity for the consortium to adapt its efforts to the needs of the scientific community.

A major opportunity to promote SILVER activities arose from an invitation for SILVER partners to participate in the AIMECS13 Conference in Taipei (October 2013).Two specific sessions were dedicated to the SILVER Project with partners presenting their work and many opportunities for meeting and discussions with industrialists arose. SILVER was given major prominence at the opening ceremony and on the agenda during the scientific sessions when SILVER partners and the coordinators presented their results and the specific details concerning the SILVER consortium and project.

Summary of the achievements of SILVER

Eight virus families were studied. From > 700,000 cell-based assays, >500 promising hits were identified and chemically modified, 20 of which were tested for Proof of concept (PoC). Target-based discovery led to the design of 186 chemical candidates one of which was tested in vivo. Of 37,000 compounds screened against purified enzymes one showed inhibitory activity in vivo and from hit explosion of chemically modified hits, 2 products are under

analysis for PoC. One licence agreement for an enterovirus inhibitor was signed with Novartis for clinical analysis and two more inhibitors are being considered for licencing. Eight patents have already been secured and the final tally is expected to reach 20 during 2015-2016. Many novel assay systems and processes have been developed and published and, at the time of reporting, 164 peer-reviewed publications are already in press and 15 PhD theses have been completed or are in the process of completion. All partners and associated scientists have participated at international conferences and many have provided advice to health agencies. In summary, SILVER has been a spectacular success with the prospect of benefits to mankind in terms of disease control, new therapeutic drugs, new scientific knowledge, education of scientists, quality of life, social impact and cost efficiency in public health.

Description of the potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of results

Socio-economic impact and wider societal implications of antiviral drug discovery

As we have shown in the annual reports of the SILVER project. The work carried out by the consortium of internationally recognised scientists under the banner of the SILVER project has produced a stunning catalogue of scientific discoveries, new data, novel protocols for in vitro assays and novel in vivo models with which to assess the quality of the products in the form of proof of concept and thus the potential for development as antiviral therapeutic agents. This summary of the impact of SILVER in the context of impact and implications will also list the specific achievements that will impact directly on the socio-economic and wider societal implications of our programme of research to discover antiviral drugs against emerging and neglected viruses. However, the significance and success of SILVER cannot be truly appreciated without briefly placing SILVER in the context of disease prevention and control.

Brief history of achievements in medical science leading to the SILVER viral drug discovery project

The concept of disease prevention and control has existed for thousands of years, in a wide range of different cultures. Indeed, treatments such as "powdered smallpox scabs taken as snuff" in ancient Greece may sometimes have worked but the mechanisms of action of the remedies were not usually understood and treatment often led to the disease rather than its prevention. Therefore, in terms of social impact and societal implications the remedies were most likely rejected by the community because the risk of treatment appeared greater than the risk of natural infection and resulting death.

Modern disease prevention methods, based on scientific analysis and therefore a "more reassured society" really commenced from the late 1700s, when Edward Jenner noticed that milk maids rarely contracted smallpox, the scourge of the time. He brilliantly deduced that exposure to the disease cowpox that appeared as pox on the udders of the cows resulted in minor infections, usually on the hands of the milk maids and this appeared to prevent them from developing smallpox. Jenner, subsequently considered to be the "father of immunology" carried out his first "clinical trial" on an 8 year old boy in 1796. He inoculated the boy with pus taken from the udder of a cow suffering from cowpox and is recorded as having demonstrated that the boy was immune to smallpox, the most deadly and common virus disease ever known to mankind. The word vaccine was derived by Jenner who took it from the Latin vacca which means cow. At first, members of the clergy claimed it was repulsive and ungodly to inoculate someone with material from a diseased animal but Jenner continued his investigations and eventually the logic and proof of his studies won over the majority of sceptics and thus the concept of a vaccine against smallpox had been established. Two hundred and eighty four years later (1980) following the administration of the vaccine against smallpox, his amazing discovery resulted in the announcement by the WHO that smallpox was officially eradicated from the World. Other scientists subsequently made equivalent exciting discoveries, Louis Pasteur and Émile Roux pioneered rabies vaccine, in 1885 and Alexander Fleming discovered the first anti-bacterial agent in 1928 which subsequently became the antibiotic Penicillin. However, the era of drug discovery for treatment of viruses didn't originate until 1957 following the observations of Alick Isaacs and Jean Lindenmann who identified a protein that inhibited viruses which they called "Interferon".

From 1957 onwards, drug discovery for treatment of virus infections became a major part of modern medical research although progress was initially very slow because technology-based molecular and structural biology were only in their infancy. However, the discovery and development of restriction enzymes for genetic modification (Salvador Luria, Giuseppe Bertani, Werner Arber, Matthew Meselson, Daniel Nathans and Hamilton Smith) heralded the beginning of a paradigm shift in medical sciences. Subsequently, the combination of technological improvements in biochemistry, molecular structural studies including protein/RNA complexes and anomalous data resolution, recombinant virus technology, genomics, medicinal chemistry, bioinformatics and high throughput screening methodologies, had evolved to the point at which scientists could set realistic targets and timeframes for antiviral drug discovery research programmes.

In November 2004 an EU 6th Framework Genomics project entitled VIZIER was initiated. Since, the common strategies used for the development of antiviral drugs are mainly based on the knowledge accumulated through studies of virus genetics and structure, the aim of VIZIER was to perform ground-breaking studies on the identification of potential new drug targets against RNA viruses through comprehensive structural characterization of the replicative machinery of a carefully selected yet diverse set of viruses. RNA viruses (ie viruses that do not have a known DNA stage in their replicative cycle), were chosen because during the past century or more, the major proportion of all emerging viruses were RNA viruses.

(Why and what are the potential consequences?)

Most emerging viruses are RNA viruses largely because replication of the viral RNA is error-prone as their genomes are too small to encode proofreading enzymes. Thus, RNA virus populations contain a myriad of closely related but genetically different variants or strains providing the viruses with the ability to adapt rapidly, by Darwinian selection, to the environmental conditions in which they have to survive. The consequences of this genetic variability, when combined with the impact of anthropology (increasing population density through urbanisation, poor living standads eg refugee camps, increasinghuman mobility and commercial transportation….) frequently result in novel or known sylvatic viruses emerging to cause outbreaks of disease that in some cases become globally distributed. Two relatively recent examples are SARS coronavirus and chikungunya virus; this comment does not ignore Ebola virus which attracted global, however, Ebola virus did not spread globally. It must also be emphasised that HIV, influenza, and hepatitis virus are deliberately excluded from these comments because they have been adequately covered by global funding and vaccines and drugs are already being used to control these RNA virus diseases.

The VIZIER project was a major success with more than 2000 genomic sequences being resolved, hundreds of relevant recombinant proteins were synthesized, and more than eighty crystal structures of viral replicative enzyme domains, ie potential antiviral drug targets, were discovered. VIZIER therefore served as the ideal platform for a dedicated viral drug discovery programme. However, the socio-economic and societal implications of the work performed in VIZIER could only really become apparent if the subsequent project, ie SILVER (drug discovery of emerging and neglected infectious diseases) was a success. As will be shown below, SILVER has been an enormous success and this justifies the intensive studies and impressive achievements of VIZIER on which SILVER was based.

The scientific and socio-economic justification for drug discovery in the face of the obvious success of vaccines, to control virus diseases, comes partly from the need for medical treatment to be directly available when viral epidemic outbreaks arise and partly from the recognition that some viruses, for example HIV, compromise the immune system when they

infect their host. In other words, appropriate vaccines can protect humans against most, but not all, RNA viruses. However, vaccines cannot normally be relied upon to treat patients already infected by pathogenic viruses. On the other hand, appropriate antiviral drugs can treat infected patients and aid their recovery from infection. Thus, in the case of infected patients with clinically apparent disease drugs become the only direct means of health control, although when clinical symptoms are apparent supportive medicine is also an important necessity.

Another often neglected societal implication of emerging highly pathogenic viruses is the threat of bio-terrorism either directly (ie the use of biological weapons) or indirectly via dual use (ie the potential for scientists conducting and disseminating the results of their research with highly dangerous pathogens). Indeed, whilst the SARS outbreak drama¬tically demonstrated how high the economic cost of an epidemic caused by an emerging virus can be, the societal implications of such a virus being used for bio-terrorism do not appear to have been considered. If they have been considered it has not been reported openly to the general public.

To address the types of issues described above in the context of emerging and neglected viruses, the EU announced a Call under the 7th Framework Programme – Health (http://ec.europa.eu/research/health). The SILVER project was conceived in response to this Call to develop a drug discovery programme to identify inhibitors of RNA viruses that fall into two classes; priority viruses (ie viruses in the families Flaviviridae, Picornaviridae and Paramyxoviridae) and other neglected and/or emerging viruses which include coronaviruses, alphaviruses, noroviruses, bunyaviruses, lyssaviruses and arenaviruses. A consortium of scientists was assembled with expertise in drug discovery, crystallography, biochemistry, recombinant virus technology, enzymatic assay development, medicinal chemistry, virology, bioinformatics and structure resolution. A pipeline strategy was developed for discovery and problem resolution. The project comprised six work packages: (i) cell-culture based screening of compound libraries and mechanism of action studies, (ii) structure-function analysis of viral enzymes in replication complexes, (iii) structure and fragment-based drug design and hit-to-lead chemical modification, (iv) proof of concept (PoC) analysis incorporating toxicity studies and in vivo models, (v) route to market and licencing to pharma (vi) project management. An outbreak pipeline and contingency funds were included in preparedness for emerging viruses. This proved fortuitous as MERS coronavirus, chikungunya and Ebola virus all emerged during the SILVER project.

During the course of the inhibitor screening project, eight virus families were studied. More than 700,000 cell-based assays were carried out resulting in more than 700 promising hits being identified and chemically modified/optimized. From this large panel of modified hits, the 20 most promising were tested for their activity in Proof of Concept assays. In separate but collaborative target-based discovery for hits, 186 chemical candidates were designed (based on the known structure) one of which was considered to have high potential was tested in vivo. Additionally, of 37,000 compounds screened against purified enzymes one showed impressive inhibitory activity in vivo and from hit explosion of chemically modified hits of this product, 2 are currently undergoing analysis for Proof of Concept.

The results of these detailed analyses have led to a licence agreement for an enterovirus inhibitor being signed with Novartis for clinical analysis. Two more inhibitors are being considered for licencing. Eight patents have already been secured for promising inhibitors and the final estimate is that SILVER will probably have filed 20 patent applications before the end of 2016. Impressively, many novel assay systems and processes have been developed and published. These new processes will enable future screening and activity analyses to be carried out more efficiently. At the time of reporting these successes, 164 peer-reviewed publications are already in press or published and 15 PhD theses have been completed or are in the process of completion. All partners and associated scientists have participated as

invited speakers or as regular participants at international conferences (eg AIMECS13, ICAR, ICAV, ASGM and others), and many have provided advice, sequence data and even reagents and diagnostic kits to health agencies (see, for example the MERS CoV report) in the face of emerging diseases. Some SILVER partners are still involved in research to discover and test inhibitors of Ebola virus. Partners, including the coordinator, are members of Advisory Committees and/or projects concerned with emerging viruses such as Ebola in West Africa, MERS CoV in the Arabian Peninsula and the globally dispersed Chikungunya virus.

Therefore, in the context of the socio-economic impact, societal implications, dissemination and even dual use, the SILVER project and the members of the SILVER consortium have contributed directly and enormously to each of these aspects. Publications by SILVER partners of successes related to drug discovery will continue to appear in peer-reviewed journals. The advisory activities together with technical backup, protocols, reagents, education, and new projects will also continue during future months. Thus, the work of SILVER and the related activities of partners have already demonstrated the potential to provide new therapies for emerging and neglected virus diseases and will demonstrate more as additional patents are filed. In terms of economic considerations and societal implications, Ebola was a striking example of the immense costs to the developed countries required to assist in controlling disease in developing countries. In terms of human suffering due to lack of health infrastructures, logistics and treatments, emerging viruses continue to challenge our capacity to eliminate these threats. Over a period of time, mankind will benefit from the new potential inhibitors that have been identified during the SILVER project and hopefully more inhibitors will be discovered as the work continues.

It is clear that the work of the SILVER scientists must continue. There are clearly many more potential antiviral therapeutic agents to be discovered and the increased technical capacity, knowledge, reagents and products gained from the SILVER project will all contribute to our ability to treat and therefore control emerging diseases as and when they arise in the future. Indeed, the fears that arise in the face of morbidity, mortality and sequelae due to emerging epidemic viruses can be significantly reduced if more time, more effort and more funding is provided to discover many more safe, effective and ultimately broad-spectrum antiviral drugs of the type to which we have alluded in SILVER.

In summary, SILVER has been a resounding success with the prospect of benefits to mankind in terms of disease control, new therapeutic drugs, new scientific knowledge, protocols, assays, dissemination of this information, education of scientists, quality of life, social impact and potential cost efficiency in terms of public health.