

**The development of a new fusion inhibitor for the
treatment of HIV**

Dr Killian Quinn

Department of Infectious Diseases

Wright-Fleming Institute

Imperial College London

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Declarations

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Abstract

Since the introduction of combination antiretroviral therapy (ART), there has been a dramatic improvement in the prognosis of people living with HIV. Indeed, data from numerous cohorts now show that for people commenced on ART at high CD4 counts and who are retained in care, life expectancy is similar to matched HIV-negative people. Consequently, the number of years an individual diagnosed with HIV can expect to be treated with ART is set to increase markedly.

With the emergence of an older population, polypharmacy and drug-drug interactions are now more prominent clinical problems in the management of HIV. Furthermore, as the number of years an individual spends on ART increases, treatment fatigue leading to suboptimal adherence and antiretroviral resistance are frequently observed. As such, the development of long-acting parenteral antiretroviral drugs with low propensity for drug-drug interactions, low toxicity and high genetic barriers to resistance is highly desirable.

Fusion inhibitors are a class of antiretroviral drugs which display limited systemic toxicities and few drug-drug interactions. They are rarely used, however, owing to injection site reactions associated with their delivery. C34-PEG₄-Chol is a novel fusion inhibitor derived from the lead molecule, C34, but modified with the addition of polyethylene glycol (PEG) and cholesterol (Chol). With addition of cholesterol, potency of the drug is enhanced by concentrating it in cell membrane domains where viral fusion occurs. Moreover, the addition of cholesterol has been shown to markedly enhance its circulatory half-life in rodents such that it may potentially act as a long-acting antiretroviral drug in humans with infrequent subcutaneous injections.

In this thesis, I assess the antiretroviral resistance pattern of C34-PEG₄-Chol *in vitro*. An adaptive clinical trial design was simulated to examine options of optimising the output of useful pharmacokinetic data after administration of single doses of C34-PEG₄-Chol to HIV-positive men. Finally, I undertook a ‘first-in-man’ study to examine the pharmacokinetic profile of C34-PEG₄-Chol and potential of this agent as a long-acting antiretroviral drug.

My work demonstrates the emergence of mutations to C34-PEG₄-Chol via novel pathways within the heptad repeat (HR)-1 domains of gp41. Compensatory mutations were also

observed within the HR-2 domain. The phenotypic effect of a HR-1 mutation was demonstrated following generation of mutant viral clones and assessing viral entry into cells in the presence of drug. An adaptive clinical trial design has shown, through simulations, to lead to more efficient allocation of trial participants across potentially therapeutic doses of the drug. Following a 'first-in-man' study, a promising pharmacokinetic profile was characterised which showed an extended drug half-life several fold above the 90% inhibitory concentration of the drug for over 4-days. This is the first time a cholesterol-endowed antiviral peptide has been entered into clinical trials in humans and has demonstrated the potential value of cholesterol-endowed peptides drugs in the field of various infectious diseases such as HIV, influenza and paramyxoviruses.

Chapter 1

General introduction

1.1 HIV in the modern antiretroviral therapy era

1.1.1 The changing epidemiology of HIV disease

The human immunodeficiency virus (HIV) is a retrovirus in the family *Retroviridae*, subfamily *Lentivirinae*, and genus *Lentivirus*. To date, two distinct viruses, HIV-1 and HIV-2 have been identified; while HIV-2 has remained geographically focused in West Africa, HIV-1 predominates globally. This thesis refers to HIV-1 unless otherwise stated. The United Nations estimates that, by the end of 2014 there were an estimated 36.9 million people (34.3-41.4 million) living with both viruses worldwide, of whom 25.8 million (24.0-28.7 million) were living in Sub-Saharan Africa [1]. Although a decline in incident infections has been reported globally, the number of people living with HIV continues to rise, largely due to antiretroviral therapy (ART) programmes in regions of high prevalence. Moreover, within industrialised countries with ready access to ART, there is now mounting evidence that, for individuals in their early twenties starting on treatment above a CD4 threshold of 350 cells/ μ L, a life expectancy approaching or equivalent to the general population can be expected, although differences by sex, race, HIV and transmission risk group remain [2]. Consequently, with improvements in life expectancy worldwide, there has been a gradual upward shift in the median age of those living with HIV over the last decade. Taking the United Kingdom (UK) as an example, in the period between 1996-2008, the life expectancy of people living with HIV has been estimated to have increased by over 15 years [3]. As a result of the increasing longevity afforded by combination ART (cART) and increasing diagnoses in people over fifty years, by the end of 2013, Public Health England (PHE) had reported that around 1 in 4 of those attending clinics for HIV care within the UK were over the age of 50 and estimate that absolute numbers of those living with HIV in the UK have now risen to around 107,800 (95% credible interval 101,600 – 115, 800) [4]. Similar trends have been observed in North America, where estimates from the Centre for Disease Control (CDC) indicate that up to 33% of patients attending for care in the United States in 2009 were over the age of 50 and it is projected that around 50% of HIV infected individuals will be over 50 years by 2015 [5]. Although data are more limited, an increasing prevalence of HIV infection in individuals over the age of 50 is also being recognised outside Europe, North America and Australasia. It has been projected that with scaling up of ART in some Sub-Saharan African countries, over 60% of those on ART will be over 50 years of age by 2040 [6].

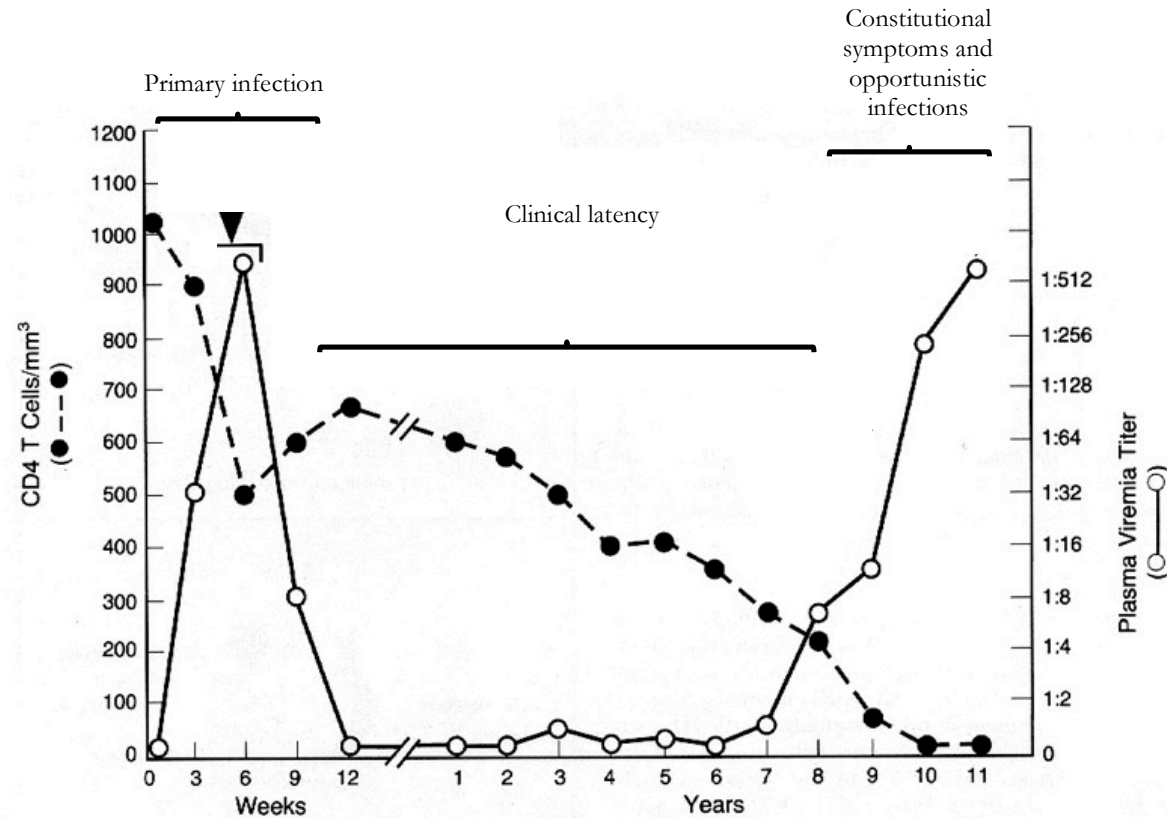
1.1.2 The traditional model of the natural history HIV infection

Descriptions of the natural history of HIV infection have previously been divided into three sequential clinical phases: 1) primary HIV infection (PHI) 2) asymptomatic chronic disease, and 3) symptomatic chronic disease. Arbitrary distinctions between the phases of infection have been based around descriptions of symptomatic seroconversion illness and subsequently stratified according to the risk of development of opportunistic infections at lower CD4 counts. However, with the results from the Strategic Management of Antiretroviral Therapy (SMART) study and, more recently, the Strategic Timing of Antiretroviral Therapy (START) study both demonstrating clinical progression and risk of non-AIDS related illnesses at all CD4 counts, it is arguable if a truly asymptomatic clinical phase can be described [7, 8].

Although estimates of between 23% and 92% have been reported [9], consensus dictates that following initial infection, between 50% and 90% of individuals experience symptoms associated with PHI. While often clinically unidentified, the syndrome of PHI is characterised by fatigue, myalgia, fever, pharyngitis, rash, mouth ulcers and generalized lymphadenopathy. Symptoms typically resolve within about 14-21 days of onset. It is perhaps as a consequence of the non-specific and often mild nature of symptoms of PHI that, even in Genitourinary Medicine (GUM) clinics where HIV is routinely tested for, around 52% of PHI went clinically unrecognised when subsequently identified as PHI on HIV avidity assays [10]. In conjunction with the clinical syndrome described, PHI is characterised by a dramatic depletion of CD4 positive T-cells from mucosal sites, most notably from the gastrointestinal tract where activated effector memory T-cells expressing high levels of the CD4 receptor and CCR5 co-receptor predominate. With ongoing replication within CD4 positive T-cells of lymphatic tissues, there is a rapid evolution of HIV viraemia [11]. While less readily quantifiable, it is now accepted that concurrent to HIV viraemia, seeding of a HIV reservoir occurs, most notably within the CCR5-positive, CD4-positive cells of the central nervous system, genital tract and, more widely, within lymphoid tissues [12]. Co-incident with massive viraemia, there is usually a profound drop in the peripheral CD4 lymphocyte count and a massive and irreversible loss in T-cell mediated immune function, occurring within 2-6 weeks of infection [13]. Upon establishing cell-mediated responses, plasma viral load tends to decline, resulting in a reciprocal partial restoration in peripheral CD4 count [13].

Following resolution of the PHI illness, highly variable disease progression rates ensue, with progression categorised as rapid, intermediate (where AIDS defining illness occur within 3 years or 3-10 years, respectively) and long-term non-progression (no AIDS defining illness or significant change in absolute CD4 lymphocyte count for > 10 years). This is depicted schematically in **Figure 1.1.1** [14]. It is estimated that around 70–80% of individuals experience intermediate disease progression in which there is a progressive and gradual rise in plasma viral load, decline in the absolute CD4 lymphocyte count and development of AIDS-related illnesses within 6–10 years of acquiring HIV [14]. On average, a peripheral CD4 count decline between 80 and 100 cells/ μ L per year is typical. Although other factors, such as low absolute numbers of HIV-specific CD8+ T-lymphocytes, diversity of cytotoxic lymphocyte responses, HLA markers, cytochrome expression, patient age and up-regulation of CD38 expression have all been strongly associated with clinical disease progression, decline in CD4 count and rise in HIV RNA plasma viral load remain the most significant predictors of disease progression [14]. While the overall risk of AIDS-related illnesses and death increases dramatically with CD4 counts below 200 cells/ μ L, it is now established that there remains an elevated standardized morbidity ratio at all CD4 count strata, and that standardised mortality ratios only normalise when ART is initiated in individuals with a nadir CD4 count above 500 cells/ μ L [15]. Although progressive decline in peripheral CD4 positive T-cells and rising HIV RNA levels remain clinical hallmarks of progressive disease, the association between HIV-induced immune activation and chronic inflammation, even in the presence of effective ART, are now recognised as major contributors to the ongoing disease state.

Figure 1.1.1: Schematic of the traditional model of untreated HIV Infection



The schematic shows that during primary HIV infection, as a result of ‘unchecked’ HIV replication there is a resulting massive HIV viraemia with concurrent widespread seeding of virus into reservoir sites. There is a simultaneous sharp decrease in the number of CD4 positive T cells in peripheral blood. As cell-mediated immunity develops, there a decrease in detectable viremia followed by a prolonged period of clinical latency. The CD4 positive T-cell count gradually declines during the following years, until it reaches a critical level below which there is a substantial risk of opportunistic diseases.

Adapted from: Pantaleo G et al. N Engl J Med 1993; 328:327-335

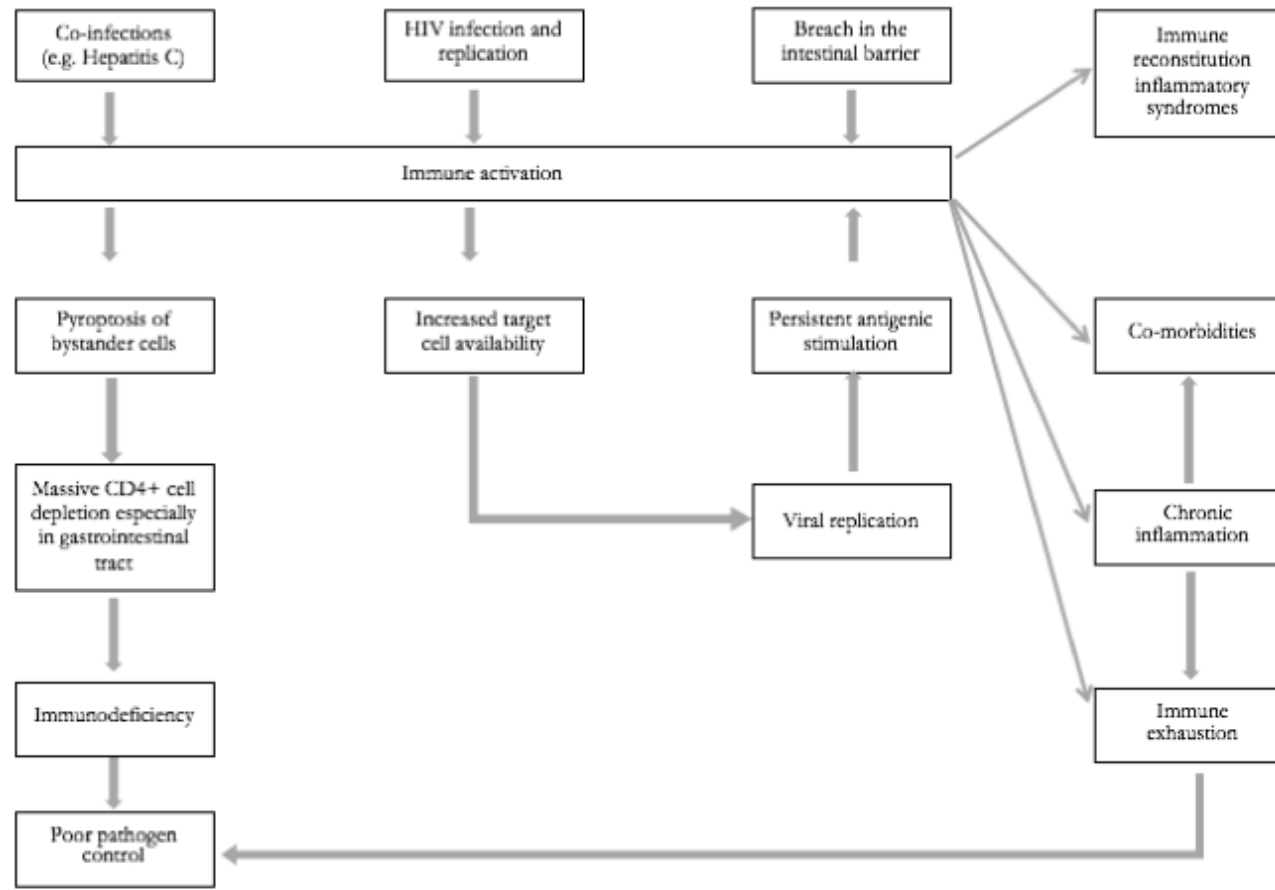
1.1.3 The evolving model of HIV infection

As morbidity and mortality from AIDS-related illnesses have undergone a sharp decline in countries with ready access to ART, the paradigm of HIV-disease is increasingly focused on the management of disease traditionally associated with ageing. Although lifestyle factors undoubtedly contribute to the increased prevalence of non-AIDS defining illnesses in HIV positive patients (e.g. cardiovascular disease and neurocognitive decline), there is evidence to suggest ongoing effects of chronic inflammation and immune activation, even in individuals who maintain HIV RNA suppression below the limits of quantification. Immune activation, driven by antigenic stimulation from HIV infection *per se* and microbial translocation from the gut, is thought to begin within days of initial infection. In particular, breaches of the mucosal integrity from depletion of mucosal associated lymphoid tissues (MALT) persist to a greater or lesser extent throughout the course of chronic infection leading to chronic inflammation. The process is characterized by a dysregulation in activation and pyroptosis of CD4- and CD8-positive T-cells, as well as natural killer cell, B-cells and monocytes. In concert, there is an increased availability of target cells which act as host cells for viral replication. High levels of proinflammatory cytokines are observed in both plasma and lymphatic tissues and are thought to contribute to a process of programmed cell death and immunosenescence. High levels of expression of interleukin (IL) -6, IL-8 β and tumour necrosis factor (TNF)- α have been observed from early in the course of HIV infection and contribute to chronic inflammation. Continuous exposure to antigen from productive HIV infection, gut microbial translocation and reactivation of latent infections (most notably cytomegalovirus and Epstein-Barr virus), have all been implicated in driving cytokine secretion. In addition, it has been postulated that that HIV gene products themselves can directly activate lymphocytes and macrophages and induce release of proinflammatory cytokines [16, 17].

With activation of immune cells, there is, in turn, increased transcription of integrated provirus such that a vicious circle ensues whereby immune activation results in increased viral production, leading to increased infection of new target cells and resulting in further release of proinflammatory cytokines and a progressive exhaustion in immune function and immune resources (**Figure 1.1.2**) [17]. It follows that with suppression of productive HIV infection, markers of immune activation decline upon initiation of ART in HIV-positive individuals, but remain elevated relative to HIV-negative matched controls. Moreover the up-regulation of such markers following treatment discontinuation has been

positively correlated with excess morbidity and mortality from the SMART (Strategies for Management of Antiretroviral Therapy) study [8]. Further analyses of data from both the SMART and ESPRIT (Evaluation of Subcutaneous Proleukin in a Randomised International Trial) studies of individuals on ART with suppressed viraemia and stratified according to CD4 counts, showed an elevated standardized mortality ratios (SMR 1.77, 95% CI 1.17 to 2.25) in individuals with CD4 counts 350-500 cells/ μ L. This only normalized with CD4 counts above 500 cells/ μ L (SMR 1.0, 95% CI 0.69 to 1.40) [8, 15, 18]. In data collated on 3280 participants on suppressive ART from both studies, a total of 62 deaths were observed, with cardiovascular disease or sudden death accounting for 19 deaths (31%), followed by non-AIDS malignancy (19%), accident, suicide, or violent death (18%), non-AIDS, non-hepatitis infection (10%), and liver disease (8%), while AIDS-related deaths accounted for only 2 deaths. Even among younger individuals with sustained viral suppression and CD4 counts between 350 and 500 cells/ μ L, there may be premature senescence driven by chronic inflammation, so that individuals with HIV present earlier with diseases associated with ageing.

Figure 1.1.2: The cycle of inflammation in HIV infection



Causes and consequences of HIV-associated immune activation are shown. *Adapted from: Roeder JM et al. [Curr Opin HIV AIDS](#). 2015*

1.1.4 The interplay between ageing and HIV disease progression

Recognition of senescent medical problems as part of the HIV disease complex have gained recognition in contemporary HIV medicine due to effective ART slowing and often, at least partially, reversing immunodeficiency. In the pre-ART era, this would have led to earlier death. Consequently, people living with HIV (PLWHIV) on effective ART are now living longer, but presenting earlier with diseases associated with ageing. Even in those with suppressed viraemia, the so-called frailty phenotype has been recognized to occur earlier in PLWHIV. Hence, the biological age may not reflect the chronological age. The frailty phenotype has been defined by unintentional weight loss, slowed motor performance, reduced physical activity and reduced power grip [5]. Although this phenotype is certainly less frequently observed in the era of cART, this has been superseded by the emergence of other HIV-associated non-AIDS conditions. Such conditions, likely driven by a combination of chronic inflammation, immune activation and lifestyle factors (e.g. increased smoking and recreational drug use), are traditionally associated with increasing age and most notably include atherosclerosis (leading to cardio- and cerebrovascular disease), osteopaenia, neurocognitive decline, non-AIDS related malignancies and metabolic disorders. In addition to appearing earlier, these conditions occur with increased frequency in PLWHIV and, as an ageing HIV population emerges, an additive effect between physiological senescence, lifestyle factors and HIV may be observed.

Following the results of the START study indicating the morbidity and mortality benefits of cART for all patients irrespective of CD4 count, all international HIV treatment guidelines have now aligned to recommend universal ART [7]. Consequently, further improvements in life expectancy may be expected and the number of years an individual diagnosed with HIV can expect to be treated with ART is set to increase markedly [19]. In addition to the emergence of senescent medical conditions in an ageing HIV infected population, the additive effects of treatment toxicities on complications of long-standing HIV infection have, therefore, become increasingly important. Additionally, the co-prescribing of ARV drugs along with medications for management of other medical problems has become a concern for drug-drug interactions (DDIs). As such, there is an ongoing need for new ARV medications with low toxicity profiles and low propensity for DDIs.

1.1.5 The need for new antiretroviral drugs

1.1.5.1 Toxicities

Early antiretroviral (ARV) drugs, although they consistently reduced AIDS-related deaths, were associated with severe toxicities; inhibition of mitochondrial DNA polymerase by thymidine analogues commonly resulted in a range of mitochondrial toxicities, including lipodystrophy [20]. Even following discontinuation of thymidine analogues, the process of lipodystrophy remains irreversible, resulting in sustained metabolic complications, raised cardiovascular risk profile and often significant psychological morbidity associated with irreversible fat redistribution [21]. Severe dyslipidaemias indirectly increasing cardiovascular risk and leading to pancreatitis precluded the use of early protease inhibitors (PIs) such as nelfinavir or saquinavir [22]. The use of the non-nucleoside reverse transcriptase inhibitor (NNRTI), nevirapine was limited to within certain CD4 parameters owing to significant risk of severe hepatotoxicity. Although newer agents within these existing classes and the development of new ARV classes have led to improved toxicity profiles from cART, many of the most commonly prescribed medications are limited by renal, bone, liver and metabolic toxicities [23, 24]. Newer nucleoside reverse transcriptase inhibitors (NRTIs), such as abacavir, may still possess propensity for mitochondrial toxicities [25]. More notably, concerns surrounding cardiovascular disease and renal and bone disease have emerged as common clinical issues with the newer agents, abacavir and tenofovir, respectively, both of which are recommended first-line regimens across all international HIV treatment guidelines [26]. Indirectly, both PIs, such as lopinavir, and the NNRTI, efavirenz, have been linked with raised a cardiovascular risk profile through elevations in LDL-cholesterol. Concerns about osteopaenia, dyslipidaemia, neuropsychiatric side-effects with efavirenz and its direct toxicities on brain parenchyma, resulting in neurocognitive impairment remain unresolved issues. Indeed, even among the integrase strand inhibitors (InSTIs), which until now had been regarded as highly tolerable, concerns have emerged surrounding tolerability of dolutegravir, particularly in relation to neuropsychiatric side effects [27]. These issues have added to concerns about the long-term safety of some of the commonly prescribed third agents of which only around 20 years of clinical experience exist for many.

1.1.5.2 Drug-drug interactions

As an ageing HIV-positive population emerges, DDIs are increasingly recognised as a clinical challenge with older PLWHIV. Either due to metabolism through the cytochrome (CYP) P450 complex, UDP-glucuronosyltransferase 1-1 (UGT1A1) or due to being substrates for the p-glycoprotein, some ARV drugs have the potential to interact with other commonly prescribed medications. Inhibition or induction of the CYP450 pathways, interference with p-glycoprotein transporters and, less significantly, UGT1A1 enzymes, may result in profound fluctuations in concentration of both the ARV drugs and non-ARV drugs, resulting in either toxicity or sub-therapeutic dosing. Notably, ritonavir and cobicistat are strong CYP3A4 and CYP2D6 inhibitors, and from the NNRTI class, efavirenz, etravirine, and nevirapine are CYP3A inducers[28]. Only FIs have displayed a general lack of DDIs as a consequence of non-NADP hydrolysis although owing to their mechanism of delivery remain undesirable in current formulations [29]. Consequently, multiple classes of non-HIV medications, including HMG-CoA reductase inhibitors, hormonal contraceptives, glucocorticoid, antimalarial medications, antimycobacterial medications, anticoagulants, calcium channel blockers, antiepileptic medications all have high propensity for significant DDIs. Within a study from the Swiss HIV cohort, of 477 patients aged ≥ 50 years and 1020 aged ≤ 50 , those aged over 50 were unsurprisingly more likely to receive at least one co-medication compared with those ≤ 50 years (82% vs. 61%; $p < 0.001$) and were more likely to be subject to potential DDIs (51% vs. 35%; $p < 0.001$) [30]. Further studies from the Swiss HIV cohort identified that in a prospective study of 1497 ART-treated patients screened for DDIs using the Liverpool HIV drug interactions website, overall 68% (1,013/1,497) of patients had a concomitant medication and 40% (599/1,497) had ≥ 1 DDI. Of concern, among patients with concomitant medications, 2% (21/1,013) were identified as being co-prescribed a concomitant medication known to be contraindicated with their current ART and 59% (597/1,013) a potential DDI warranting either dose adjustment or close monitoring for toxicity [31]. Within the UK, data collected prospectively from 159 patients attending an HIV outpatient service identified a total of 213 potential DDIs of which 86 were defined as clinically significant i.e. serious or potentially serious on the Liverpool HIV drug interactions website. From multivariate logistic regression analyses, clinically significant DDIs were significantly associated with receipt of PIs (OR, 10.59; 95% CI, 2.86–31.87; $P < .001$), being male (OR, 3.02; 95% CI, 1.06–8.58; $P = .038$), although, surprisingly, not associated with age or receipt of NNRTIs [32]. Similar studies from the United States HIV Outpatient Study (HOPS) have shown

that in a cohort of 3,810 patients on ART with a median age of 46 and 34% over the age of 50 years, almost 40% of patients were prescribed ≥ 5 non-ARV drugs. Unsurprisingly, a significantly greater proportion of patients over the age of 50 were prescribed ≥ 5 non-ARV drugs (54%) compared with those under 50 years (32%). Of greatest concern, however, was the number of patients who were prescribed at least one ARV/non-ARV combination that was contraindicated (7%) or had moderate or high evidence of interaction (33 %) [33].

1.1.6 The need for long-acting antiretroviral drugs

1.1.6.1 Clinical experience with long-acting drugs for long term indications

Long-acting parenteral medications have a number of advantages that include limiting non-adherence to treatments, reducing pill burden, allowing a sustained release of drug and limiting variation between peak and trough doses. On the other hand, it has been argued that, especially in the case of long-acting injectable (LAI) agents, a major disadvantage may be the inability to reverse or remove an agent immediately in cases of toxicity or hypersensitivity reactions. Any new formulations need careful consideration of their safety, including the potential for a lead-in phase with an orally bioavailable parent compound as a trial of tolerability to a new ARV agent. To date, clinical experience with long-acting drug formulations for chronic indications are used in four main clinical domains, namely psychiatry and substance misuse, contraception, hormonal antagonists and drug-eluting devices. Long-acting ARV agents, capable of monthly or less frequent administration, and providing sustained release following subcutaneous or intramuscular injection have the potential to improve adherence and extend opportunities for prophylactic intervention in patient groups at risk of HIV acquisition, while simultaneously reducing the pill-burden, especially for older patients.

1.1.6.2 Long-acting injectable drugs

Depot antipsychotic medications form the largest group of LAI medications and have become a core component of the management of chronic psychoses [34]. There are now nine depot antipsychotic medications developed since the first use of fluphenazine enanthate in 1966 [35]. The main indication for LAI antipsychotics is for patients with long-term psychoses (e.g. schizophrenia) who have a history of responding well to an oral antipsychotic, but who have had sub-optimal adherence, resulting in disease relapse.

Developments in the field of psychiatry and substance misuse has lead to the use of LAI and subcutaneous implants of naltrexone, an opiate antagonist used in both alcohol and opiate misuse, again following a lead-in phase with oral naltrexone with variable success [36]. The LAI hormonal contraceptive, depot medroxyprogesterone acetate (Depo-Provera), has also been used for around forty years and remains the only injectable hormonal contraceptive available in the UK [37]. It is currently licensed for administration every 12 weeks.

1.1.6.3 Drug-eluting implants

Drug-eluting devices are medical implants which release active pharmaceutical ingredients with the aim of inducing healing in addition to a mechanical supportive effect. They are perhaps best known within the field of coronary angioplasty [38]. Coronary stents typically elute antimitotic agents (such as paclitaxel) and anti-inflammatory agents (for example, sirolimus) to prevent stent stenosis after grafting [39, 40]. Other examples have included drug-eluting wound dressings containing antimicrobial agents and growth factors and protein-eluting scaffolds for tissue regeneration [41].

1.1.6.4 Drug implants

Drug implants as a means of prolonging drug release remain among the most widely investigated. Modalities investigated include non-biodegradable devices in which drug is embedded within a polymeric matrix and made available through diffusion. Subcutaneous contraceptive implants remain the best example of these and have been in common use since 1998, allowing sustained release of etonegestrel over a three-year period. In the field of infectious diseases, ocular implants for delivery of ganciclovir have also been used to a limited extent in the past to treat cytomegalovirus retinitis as part of an AIDS-defining illness

Biodegradable implants in which the matrix and active compound dissolve slowly following implantation have also been developed and used in other branches of medicine. The most notable is Goserelin (Zoladex), a gonadotrophin releasing (GnRH) agonist used as an anti-testosterone and anti-oestrogen in the treatment of prostate and breast cancers, respectively and delivered every three months as micropellets [42].

1.1.6.5 Situations for use of long-acting antiretroviral therapy

Suboptimal adherence to cART results in persistent low level viraemia, selection of resistant viral haplotypes and sustained systemic chronic inflammation, ultimately leading to higher morbidity and mortality outcomes in HIV infected patients. Plasma HIV RNA load is now well established as the chief predictor of the risk of heterosexual transmission of HIV, where it has been recognized that transmission is rare among persons with levels of less than 1500 copies of HIV RNA per milliliter[43]. It is arguable that suboptimal adherence to cART among individuals on treatment has wider public health implications. Indeed, results from the HPTN-052 study provide compelling evidence for treatment as prevention, with a striking 96% reduction in HIV transmission amongst serodiscordant couples where the HIV infected partner starts treatment immediately in conjunction with standard safe sex practices [44].

In addition to the need for sustained adherence with cART, as the success of cART has resulted in the emergence of an ageing HIV infected population, there is increasing recognition of polypharmacy amongst older patients. Indeed, in the recently published data from the United States HOPS cohort, amongst patients over the age of 60 attending a large HIV outpatient service in California, 96% patients were taking five or more medications and 76% were taking nine or more medications [45]. Thus, long-acting ARV drugs may mitigate to some extent against large pill burdens, particularly in elderly patients.

With increased focus on the use of ARV drugs for HIV pre-exposure prophylaxis (PrEP), suboptimal adherence results in marked differences in HIV acquisition in trials involving daily combinations of tenofovir disoproxil fumarate (TDF) and emtricitabine (Truvada). Specifically, initial studies examining the risk reduction in HIV acquisition afforded by daily Truvada have shown a 44% reduction in HIV acquisition among high risk men who have sex with men (MSM) in intention to treat analyses [46]. Upon exclusion of those who were demonstrably non-adherent through pharmacokinetic (PK) analysis of plasma drug levels however, a risk reduction of up to 99% was observed in those reporting adherence for 7 days a week [46]. Within the context of the UK, the benefits of Truvada for PrEP have been demonstrated more widely within the PROUD study (Pre-Exposure Option for reducing HIV in the UK: immediate or Deferred) where an 86% reduction in HIV acquisition was observed amongst MSM who started PrEP immediately relative to a those who were deferred from starting for 1 year highlighting the potential widespread public

health benefits of a roll out program of PrEP amongst individuals at high risk of HIV acquisition [47]. Beyond the context of a clinical trial however, the sustained use of Truvada as a prophylactic intervention remains plagued by ongoing concerns of non-adherence.

1.1.6.6 Formulation of long-acting antiretroviral regimens

The dosing frequency of an ARV agent is determined by its aqueous solubility, antiviral potency and systemic clearance kinetics. The majority of oral agents are once daily formulations. Although analogies are frequently made between the development of long-acting antipsychotics and contraceptives with LAI ARV drugs, the daily oral dose of paliperidone, a typically prescribed antipsychotic, is 3-12mg while that of medroxyprogesterone acetate is 2.5-10mg, significantly lower than those of the commonly used NRTIs, NNRTIs, PIs and InSTIs [48]. Taking NRTIs as an example, development as long-acting parenteral agents has been limited by high aqueous solubility, relatively low antiviral potency, and high systemic clearance kinetics. Moreover, as prodrugs requiring activation by intracellular phosphorylation, the low efficiency of this process continues to demand high therapeutic levels and thus would require high parenteral dosing. Coupled with the need for combinations of ARV drugs for effective virological control, the objective of developing multiple drugs with sufficiently low volumes to be co-administered has been elusive.

A variety of nanotechnologies are currently under exploration to overcome difficulties with the development of long-acting formulations of some ARV classes. Most widely described has been the development of rilpivirine long-acting (LA) through a process of aseptic, wet bead milling until particles within the nanometer range are achieved leading to increased drugs solubility in aqueous media [49]. Preclinical studies of single doses of early rilpivirine LA nanosuspension administered either intramuscularly or subcutaneously to dogs and mice resulted in sustained rilpivirine concentrations in plasma. Similar results were replicated in a phase I study of HIV-negative healthy volunteers with sustained release of rilpivirine from injection sites into plasma in therapeutic ranges over 12-26 weeks [50]. It is assumed that, due to the very low solubility of rilpivirine, particles dissolve very slowly at the site of injection and allow release over an extended period after injection. Similar approaches have been used for the production of cabotegrevir LA, an InSTI developed in both a daily oral and LAI formulations. Similarly, through a process of wet milling,

crystalline active drug with particles within the nanometer range have been produced with initial phase I studies, demonstrating sustained plasma concentrations above the protein-adjusted IC_{90} for approximately 24 weeks, or longer when doses above 200 mg were administered [51]. The potential of long-acting ART became an exciting possibility following PK and pharmacodynamic (PD) studies on combinations of oral preparations of rilpivirine with cabotegrevir, which proved to be as effective as efavirenz plus dual NRTIs at 96 weeks of follow up. Indeed, in a trial of twenty HIV-negative volunteers, two consecutive doses of the combination of cabotegrevir LA and rilpivirine LA have been shown to be well tolerated and sustained plasma concentrations of both drugs within the therapeutic range at 4-weekly dosing intervals [52]. Long-acting nanosuspensions of nevirapine and atazanavir/ritonavir have been trialled in rodent studies demonstrating a proof of concept but have yet to enter clinical trials [53] [54]. Aside from nanotechnologies, a subdermal implant preparation of tenofovir alafenamide (TAF) has also been trialled in preclinical studies with beagle dogs and showed sustained release over 40 days post-implantation of the device. Consequently, subdermal TAF has been proposed as a potential means to deliver post exposure prophylaxis (PEP) and PrEP, but this could have a potential role within long-acting cART regimens. Of other classes of ARV drugs, only fusion inhibitors (FIs) have entered clinical trials as potential long-acting agents and have shown malleability in design as long-acting ARV drugs with low propensity for DDIs. They are regarded as having low potential for systemic toxicities.

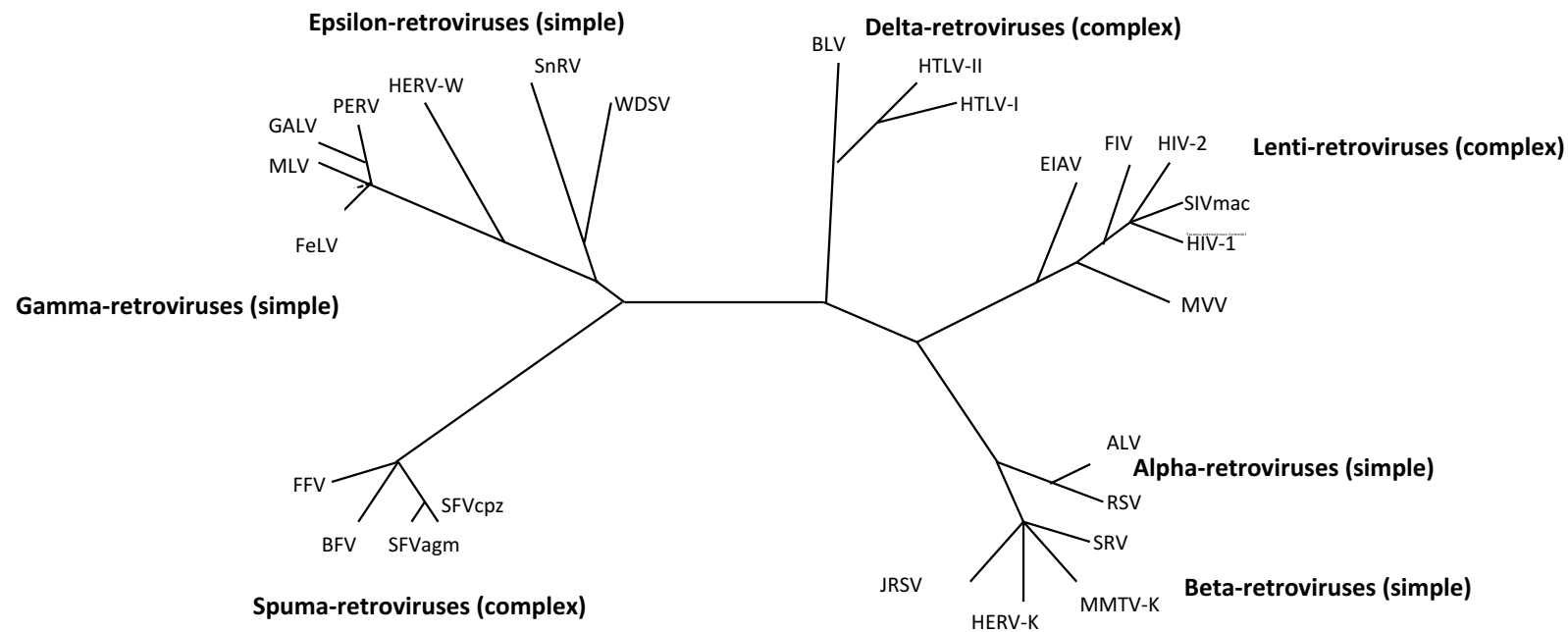
Currently, the first line regimen in all international HIV guideline committees remain a combination of three ARV drugs consisting of two NRTIs coupled with a third agent drawn from the PIs, NNRTIs or InSTIs. Despite trials of various regimens, including PI monotherapy and InSTI/PI and more recently InSTI (Dolutegravir) monotherapy, to date no NRTI-sparing regimen has proven to be a superior than dual NRTI backbone plus a third agent. As such, this conflicts with formulation problems with long-acting NRTIs in particular meaning that any LAI regimen is likely to be NRTI sparing and, thus, only utilized for specific clinical indications.

1.2 The Virology of HIV

1.2.1 Phylogeny of HIV

The phylogeny of retroviruses is that of related group of viruses that depend on reverse transcription for their replication. They are subdivided into Beta-, Spuma, Gamma, Epsilon, Delta and the Lenti- retroviruses to which HIV belongs (**Figure 1.2.1**). Phylogenetic analyses have demonstrated a close relationship to the Simian Immunodeficiency Virus (SIV) with a closer relationship existing between HIV-1 and SIV derived from chimpanzees and HIV-2 with the SIV from sooty mangabeys (**Figure 1.2.2 A**). HIV-1 is further subdivided into four distinct groups (M, N, O and P); the HIV-1 group M is the main group of viruses and is almost entirely responsible for the global pandemic (**Figure 1.2.2 A**). When represented on a phylogenetic tree, closely defined clusters, known as subtypes (A-D, F-H, J and K) or clades, exist within group M (**Figure 1.2.2 B**). In addition, circulating recombinant forms (CRFs) have been identified and these constitute a significant part of the pandemic. Subtypes and CRFs share similarities in genetic diversity and tend to predominate within defined geographic distributions. In the UK, HIV-1 Group M subtype B is most widespread.

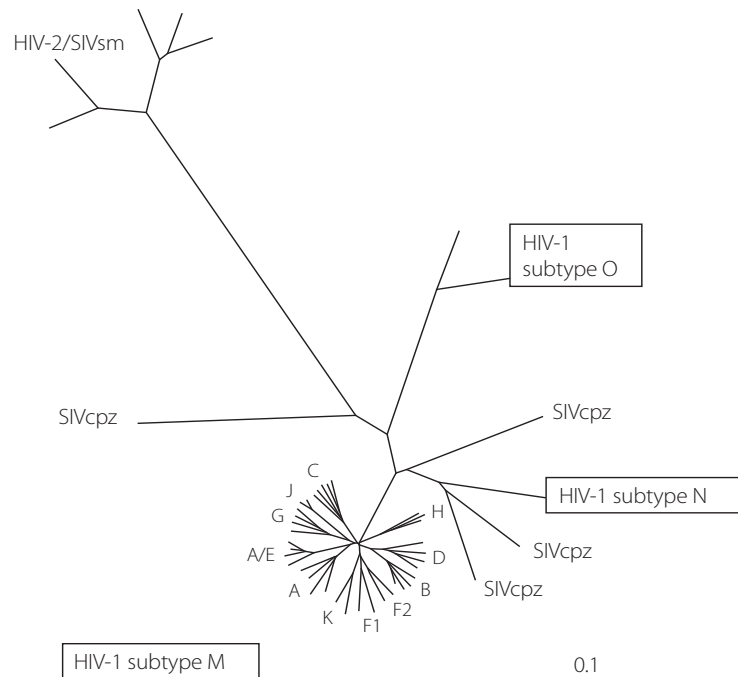
Figure 1.2.1: The Phylogeny of Retroviruses



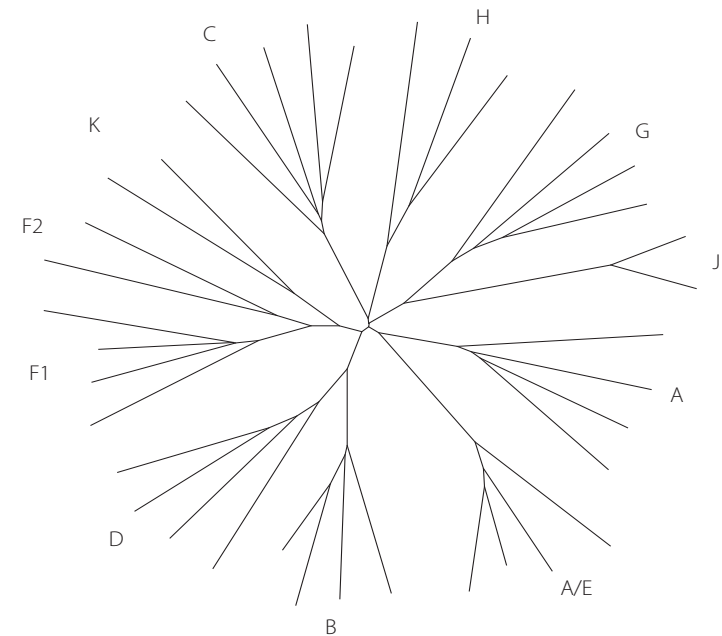
Phylogenetic tree of major retrovirus families showing relative relatedness. The length of individual lines indicates the relative similarity/difference in genetic sequence between viruses. Note the position of XMRV is shown, the dotted line indicating its position in the tree but not its sequence similarity to other virus. The human pathogens HIV-1, HIV-2, HTLV-1 and HTLV-2 are seen within the complex viruses. Adapted from: Kenyon J *et al. Br Med Bull* 2011; 98; 61-74

Figure 1.2.2: The Phylogeny of HIV

A



B

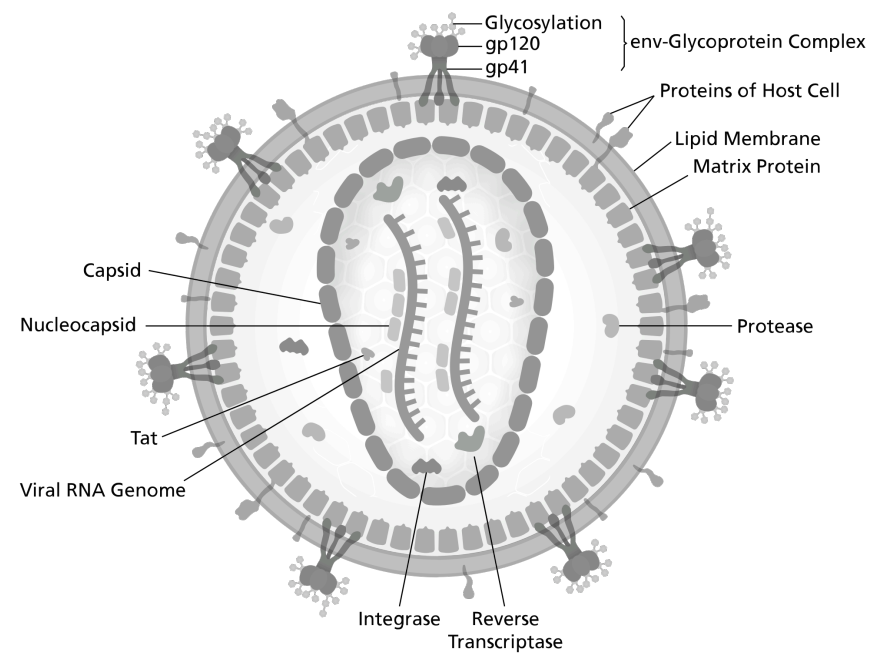


A. Phylogenetic tree using alignments of the envelope glycoproteins derived from gp160. HIV is separated into two types, HIV-1 and HIV-2. HIV-1 co-segregates with simian immunodeficiency virus from chimpanzees (SIV_{cpz}) while HIV-2 co-segregates with SIV from sooty mangabeys (SIV_{sm}). **B.** The phylogeny of HIV-1 subgroup M (which constitutes most of the global pandemic) based on an alignment of envelope gene sequences is adapted from the Los Alamos National Laboratory (USA) HIV Sequence Database. Group M strains are separated into subtypes A-K (without I and a subdivision of F into F1 and F2). Taken from: *HIV management in Australasia: a guide to clinical care (2009 Edition)*. Australian Society of HIV Medicine.

1.2.2 The structure of HIV

HIV is an enveloped retrovirus with a distinct cone-shaped capsid encasing the viral genome and surrounded by a lipid bilayer envelope [55] (**Figure 1.2.3**). Viral particles assemble and bud from host cells giving rise to an outer lipid bilayer enriched with cholesterol and sphingolipid microdomains, known as lipid rafts. It has been estimated that this viral envelope is studded with between seven and fourteen trimeric envelope proteins consisting of glycoproteins (gp)41 and gp120 subunits that are involved in the process of viral entry into host cells [56]. In addition, as a result of viral budding from host cells, host derived proteins, including major histocompatibility class (MHC) antigens and actin, form part of the viral envelope. The HIV envelope gps are derived from gp160, a parental polyprotein encoded by the *env* gene of the HIV genome [57]. Proteolytic cleavage of gp160 gives rise to both gp41 and gp120, which are arranged as trimers of three gp41 subunits non-covalently bound to three gp120 subunits on the viral surface. Together, they mediate the process of viral entry [57]. The proteins serve to anchor the virus to the target cell and facilitate fusion. Integrity of the virion particle is maintained by the presence of the p17 viral protein matrix lying immediately below the outer lipid envelope and encasing the conical viral capsid. In a mature, infectious HIV virion, the viral genome is housed within a conical capsid core made from the viral capsid protein, p24 [58]. Within the capsid reside two positive sense strands of unspliced viral genome bound tightly to the nucleocapsid proteins, p7. Nucleocapsid proteins are thought to play a key role in stabilizing and preventing digestion of the ribonucleic acids (RNA) by nucleases which encode the genome [59]. In addition, the capsid contains enzymes essential to the lifecycle of the virus, namely reverse transcriptase (RT) and integrase (while protease is held between the capsid and the protein matrix). Virus particles also package the accessory proteins derived from the genes *nef*, *vif*, *vpr* and *vpr* and two additional unpackaged regulatory proteins derived from *rev* and *tat* which function in the host cell [60].

Figure 1.2.3: The HIV virion



The HIV structure highlighting envelope proteins (gp120, gp41), lipid membrane and structural proteins making up the matrix, capsid and nucleocapsid. Enzymes (reverse transcriptase, protease and integrase) essential for the HIV lifecycle are also highlighted. The single RNA strands which make up the viral RNA genome are found within the viral capsid and are linked (not depicted within the diagram). Taken from: *Rockstroh JK et al (2016): HIV 2015/16* www.hivbook.com

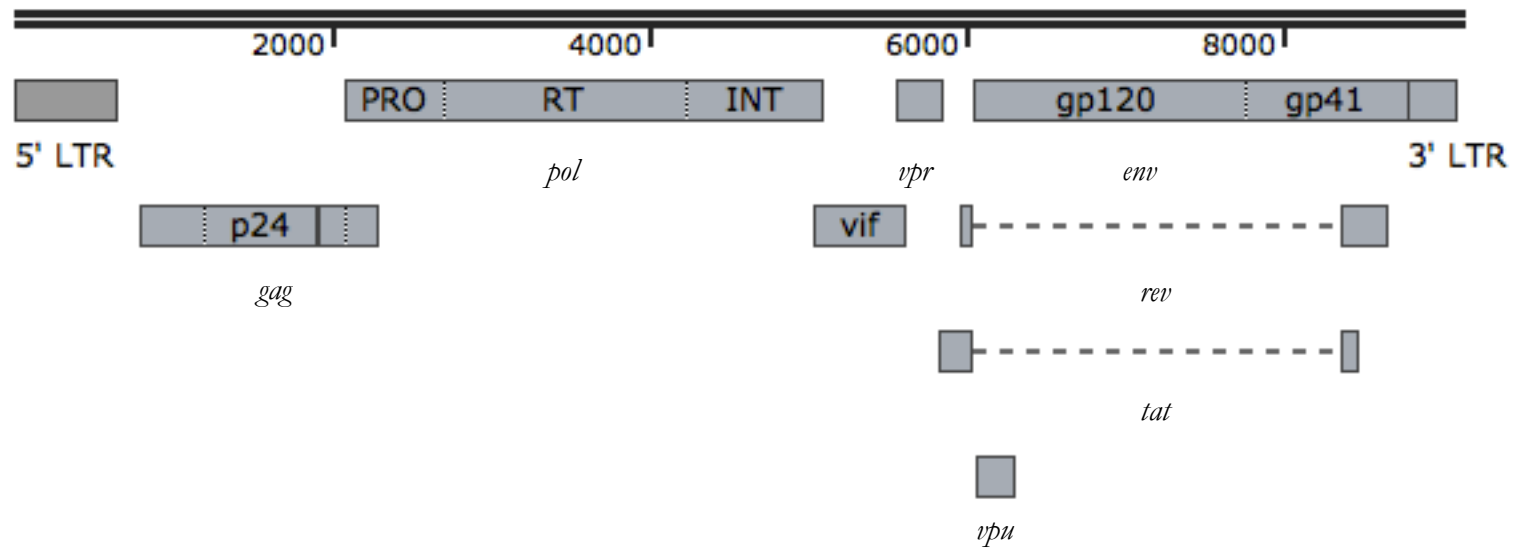
1.2.2 The HIV genome

The genetic information of HIV is stored within two copies of positive sense RNA strands containing nine reading frames. The genome itself is about 9.7 kilobases in length (**Figure 1.2.4**). Genes can be broadly divided into three main functional groups: 1) structural 2) regulatory and 3) accessory genes. Three genes, *gag*, *pol* and *env*, encode the structural proteins, expressed as polyproteins which undergo proteolytic cleavage to give rise to a total of 15 proteins involved in the HIV lifecycle and viral structure. Together, *gag* and *env* are the two genes responsible for production of proteins which are essential for the structural integrity of the virus; *gag* gives rise to the four proteins found within the viral capsid (matrix, p24 capsid, nucleocapsid and p6). Translation of *env* initially results in the formation of the gp160 polyprotein which undergoes proteolytic digestion by a cellular protease during transit to the viral membrane to form the envelope proteins (gp120 and gp41). The *pol* gene encodes the three enzymes, RT, protease and integrase that are essential for the virus lifecycle and which are not available via the host machinery. Regulatory genes, *tat* and *rev*, are responsible for modulating transcriptional and post-transcriptional steps of viral gene expression essential for virus propagation. Following integration of the HIV proviral DNA into host cells, the 5' long terminal repeat (LTR) acts as a weak promoter of transcription. After transcription, in the absence of Rev, the messenger RNA (mRNA) is spliced up resulting in only small transcripts being transported into the host cytoplasm to express the proteins Rev, Tat and Nef. The protein Tat is able to translocate into the nucleus and help activation of transcription at the 5' LTR promoter such that more mRNA is produced. Rev also activates transcription at the 5' LTR and promotes the transport of unspliced mRNA out of the nucleus thereby promoting expression of remaining viral proteins. The absence of *rev* and *tat* from the genome would therefore reduce expression of many viral proteins [61].

While their presence was previously thought to be dispensable for active infection *in vivo*, the role of the accessory genes, *vif*, *vpr*, *vpu* and *nef*, has now been more clearly defined [62]. Broadly, accessory proteins modify the cell environment to make the host cell more receptive and resilient in its ability cause survive a persistent infection, allow viral replication and ultimately allow efficient production of viral progeny [63]. None of the HIV accessory genes possess enzymatic activity. Their presence also allows evasion of various forms of host immune responses and results in redirecting host factors to act on virus specific processes. Specifically, the role of the proteins Vif, Vpu and Vpr is to recruit

proteasomes (such as cullin-RING finger ubiquitin ligases) to eliminate ARV host proteins thus antagonising the effects of ARV host restriction factors [64]. Most notably, through downregulation and elimination of the restriction factors APOBEC3G, Trim-5 α and BST-2/tetherin by Vif, Vpu and Vpr respectively, their antiviral effects are averted [65]. Nef and Vpu also downregulate CD4 through accelerating endocytosis of CD4 and Vpu also regulates the location and concentration of viral proteins on the surface of infected cells, enhances virion infectivity and interferes with MHC expression.

Figure 1.2.4: The HIV genome

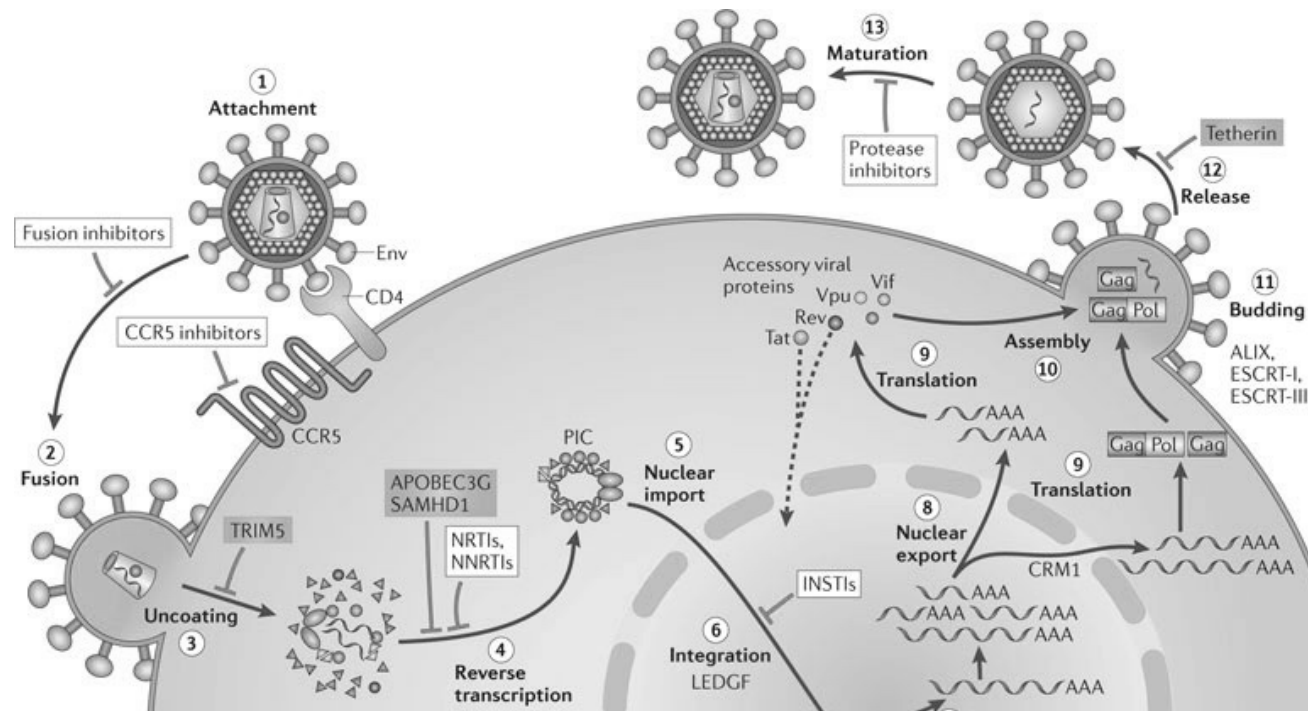


The single-stranded RNA genome of HIV encodes nine major structural and catalytic proteins by using overlapping parts of the genome; the nine genes include the structural genes (*gag* and *env*), the catalytic gene (*pol*), the regulator genes (*tat* and *rev*) and the accessory genes (*vif*, *vpr*, *vpu*, *nef*). Additionally, the nucleic acid secondary and tertiary structure performs functions independent of translation. LTR = long-terminal repeat. *Derived from GenBank: AF033819.3 created using SnapGene.*

1.2.3 The HIV life cycle and antiretroviral targets

Insights into the life cycle and structural biology of HIV have aided significantly in the development of therapeutic interventions. The application of mechanistic approaches to the development and discovery of novel antiretroviral targets have accelerated the throughput of compounds into preclinical and clinical trials, such that there are now over 30 drugs approved for the treatment of HIV [66]. Such approaches have included 1) computational molecular modeling for drug design based on the molecular structure of viral proteins, 2) optimization of pre-existing lead compounds derived from homologous enzymes and viral proteins targets and 3) high throughput screening of compounds to identify molecules which modulate the HIV lifecycle [66]. Although these have helped to identify multiple targets within the HIV life cycle for the development of ARV therapy, only four stages within the life cycle have translated to approval of agents from six classes of ARV drugs, namely viral entry (attachment and fusion), reverse transcription, integration and viral maturation (**Figure 1.2.5**).

Figure 1.2.5: The HIV lifecycle



Schematic of the HIV life cycle. Infection begins with the engagement of envelope glycoproteins with the CD4 receptor and chemokine co-receptor on the host cell membrane, resulting in mixing of the viral and cellular bilayers and internalisation of the viral capsid inside the target cell. Capsid uncoating facilitates reverse transcription and subsequently the formation of the pre-integration complex (PIC). The PIC is then transported inside the cell nucleus and is integrated into the cellular DNA aided by lens-epithelium derived growth factor (LEDGF) to form the provirus. Proviral transcription yields messenger RNA (mRNA) which serves as templates for protein production. Genome length RNA is incorporated into viral particles with translated proteins. The cycle ends with viral particle budding. FDA approved classes of antiretroviral drugs are shown within the white boxes. Adapted from: *Engelman A et al. Nat Rev Microbiol.* 2012 Mar 16;10(4):279-90.

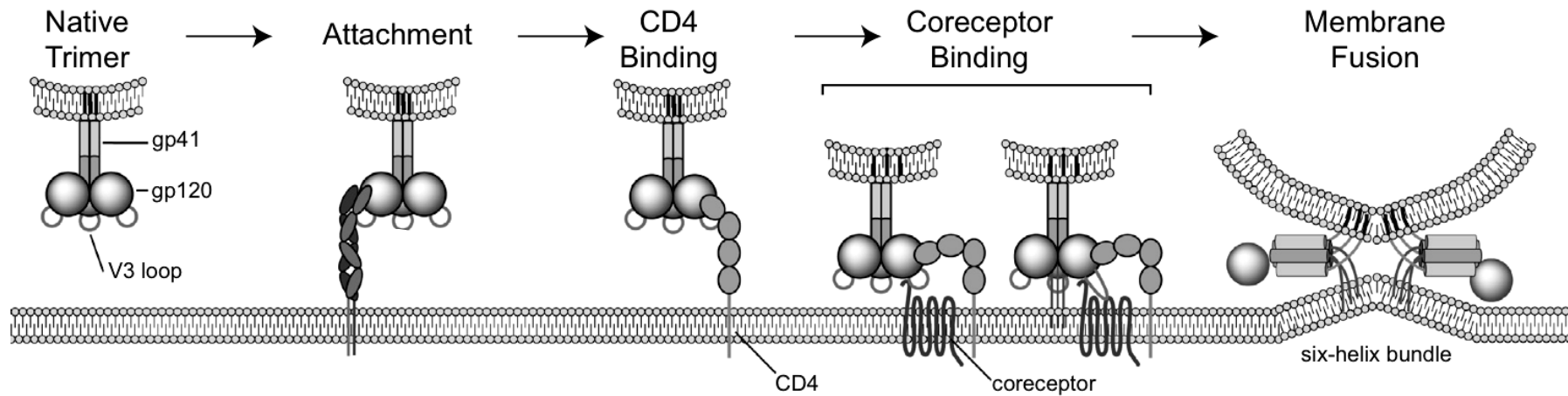
1.2.3.1 Viral Entry

The HIV life cycle can be divided into afferent, nuclear and efferent processes. HIV primarily infects CD4-positive T-cells and macrophages. The binding of an extracellular infectious virion to a susceptible CD4 expressing cell begins the afferent stage of the life cycle. The process of viral entry can be broken down into 3 main steps, namely 1) attachment of gp120 to the CD4 target cell receptor, 2) interaction of gp120 with chemokine co-receptors and 3) fusion of the viral and cell membranes [67]. Following attachment of the V3 loop of gp120 to the chemokine co-receptor, the gp41-gp120 complex undergoes a conformational change exposing the fusogenic ectodomain of gp41 [68-70]. The ectodomain consists of three essential subdomains, the fusion peptide (FP), heptad repeat (HR)-1 and HR-2 regions (sometimes referred to as N-heptad [NHR] repeat and C-heptad [CHR] repeats), the latter two of which exist as α -helices (a common secondary structure of proteins consisting of amino acid chains existing as right-handed coil conformations) [67, 69, 71-74]. Stage 3 of the process begins with the exposure and insertion of the FP into the target CD4 cell. Following insertion of the FP, three hydrophobic HR-1 α -helices fold into one another forming a thermostable left handed 'coiled-coil' bundle; formation of this triple α -helix results in the creation of hydrophobic grooves extending antiparallel across the outer surface of the HR-1 coiled-coil bundle and forming a binding interface for the complementary HR-2 subdomains. Crucially, within each of these grooves, a deep hydrophobic pocket domain (PD) is formed towards the C-terminal of the HR-1 [67-69, 71-73]. Crystallographic studies provide support that, after the formation of the α -helical bundle, the ectodomain folds into a hairpin active state such that the complementary HR-2 regions align along the hydrophobic grooves on the HR-1 coiled-coil bundle [74]. By shortening the gp41 ectodomain, the viral and cell membranes are pulled into apposition to create a fusion pore through which the viral capsid can be released and effect viral-cell fusion [69, 75]. With the formation of this 6-helical hairpin bundle (6-HB), amino acid residues on the outer surface of the HR-1 α -helices can interact with complementary residues in the HR-2 α -helices [74]. Most critically, however, stability of the 6-HB is thought to be maintained by interaction of residues within the PD of HR-1 with those from the pocket-binding domain (PBD) of HR-2 [74, 76]. The process of viral entry is thought to be completed within about 1 hour.

Viral entry represents a major target for the development of novel ARV compounds and development of entry inhibitors has been focused on the three steps described 1)

attachment inhibitors 2) chemokine co-receptor antagonists and 3) FIs. To date, however, only one drug from the cysteine-cysteine co-receptor (CCR5) antagonists [77] and HR-2-based FIs [78, 79] have been approved to target steps 2 and 3, respectively. Other molecules aimed at each of these steps have been trialed or are currently within clinical trials, including attachment inhibitors for step 1.

Figure 1.2.6: The HIV entry process



The schematic shows that gp120 and gp41 are located on the viral membrane and arranged as a trimer, while CD4 and coreceptors are located on the target cell membrane. **Attachment:** HIV entry begins with anchoring of gp120 to target cell proteins. **CD4 binding:** binding of CD4 to gp120 induces a conformational change within gp120, exposing the V3 loop. **Coreceptor binding:** Exposure of the V3 loop allows attachment of gp120 onto a chemokine co-receptor. Attachment of gp120 to the coreceptor results in a further conformational change within the trimeric complex, exposing the fusogenic ectodomain of gp41 which pierces the target cell membrane. **Membrane fusion:** As the heptad repeat (HR) domains of gp41 fold on top of each other, the viral and cell membranes are pulled into apposition, resulting in the formation of a fusion pore through which the viral capsid is emptied. Adapted from: Haqqani AA *Antiviral Res.* 2013 May;98(2):158-70

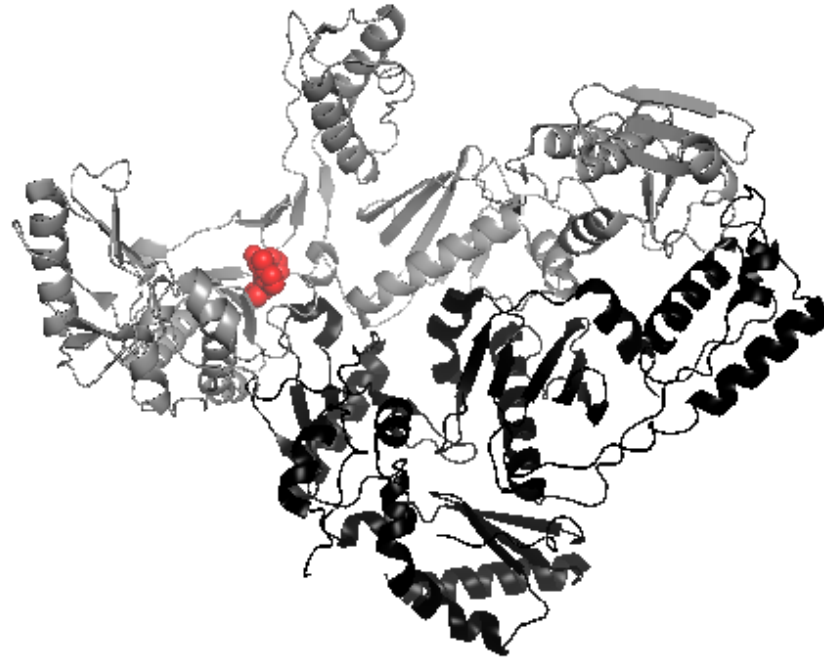
1.2.3.2 Reverse transcription

Following cytoplasmic penetration of the viral core, partial capsid dissolution releases viral RNA coupled with nucleoprotein into the host cells. Viral RT harnesses host nucleotides and initiates the process of reverse transcription of RNA into RNA-DNA duplex and subsequently double stranded DNA. RT was the first enzyme target in the development of ARV drugs and is the target for two distinct classes of ARV drug: the NRTIs and NNRTIs.

RT is an asymmetric heterodimer of two related but distinct subunits, p66 and p51 (**Figure 1.2.6**). It has two catalytic domains: the DNA polymerase active site and RNase H. After penetration of the viral capsid into the host cell cytoplasm, the RT polymerase active site undertakes transcription of single-stranded RNA into an RNA-DNA double helix. With the activity of the RNase H active site, the RNA component of the RNA-DNA double helix is digested, while the polymerase active site can then complete the synthesis of the double-stranded DNA to create a double-stranded helix which can then be integrated as proviral DNA into the host genome. Nucleotide analogues antagonistically bind to DNA polymerases, displacing host nucleotides with nucleotides deficient of a 3-hydroxy terminal and, as such, act as non-functional nucleotides. Consequently, once incorporated, because host nucleotides can then no longer attach to the incorporated analogues, DNA chain synthesis is terminated. Hence, because of this mechanism of action, NRTIs are sometimes referred to as “chain terminators”.

The activity of RT can also be inhibited through targeting the attachment of a moiety into the NNRTI binding pocket, a hydrophobic binding site adjacent to the polymerase active site of the p66 subunit; through binding of moieties into the pocket, there results a reduced conformational flexibility of the enzyme such that reverse transcription and polymerisation of single stranded DNA is blocked.

Figure 1.2.7 Three dimensional representation of reverse transcriptase



Three dimensional representation of HIV reverse transcriptase with the two subunits p51 (light grey) and p66 (dark grey); nevirapine is represented as a red molecule adjacent to the active sites of the enzyme. Created using PyMOL, protein databank sequence 1REV. Adapted from: *Ren J et al.*

Structure, 1995 Sep 15;3(9):915-26

1.2.3.3 Integration

Following completion of reverse transcription, the double-stranded DNA forms either stable DNA circles within the host cell cytoplasm or is transported to the nucleus and randomly integrated into the host-cell genome. The integrase incorporates transcribed viral DNA into the host cell genome in a two stage process, namely 3'-processing and DNA strand transfer. The 3'-processing begins within the host cytoplasm with the attachment of viral integrase to the reverse transcribed viral DNA, resulting in the formation of a pre-integration complex (PIC). During 3' processing, integrase recognises specific sequences within the viral LTR and cleaves terminal dinucleotides from each of the 3' ends of the viral DNA to yield chemically-reactive hydroxyl groups within the viral DNA.

Following 3'-processing, the PIC is actively transported inside the cell nucleus. It is hypothesised that the PIC harbours factors that promote active nuclear import, as its size is larger than the passive diffusion limit for nuclear pores [80]. The strand transfer reaction is catalysed by the intasome, a tetramer of integrase and viral DNA, in a process whereby integrase subunits cleave the top and bottom strands of the target DNA phosphodiesterase backbone in a staggered fashion and subsequently utilise reactive hydroxyl groups within the viral DNA to integrate into host DNA [81].

After disassembling of the strand transfer complex, cellular enzymes then repair the recombinant DNA to give rise to an integrated provirus flanked by the host genome. Both 3' processing and strand transfer are divalent cation-requiring trans-esterification reactions catalysed at a single active site in the core of integrase [82]. To date, the crystal structure of HIV integrase has not been elucidated but through biochemical assays it has been determined that integrase inhibitors do not inhibit 3'-processing and selectively block strand transfer and as such, are more correctly termed InSTIs.

1.2.3.4 Transcription

Following integration into the host genome, the provirus acts as a transcriptional template for full-length viral RNA and messenger RNA (mRNA) which, in turn, is translated to produce essential viral proteins. As with all retroviruses, the LTR acts as the viral promoter for transcription [83]. Immediately after integration, short unspliced mRNA encoding the regulatory proteins, Tat and Rev, is produced. Tat regulates transcription by stimulating elongation of mRNA from the LTR promoter [83]. Rev transports the mRNA molecules

encoding the structural proteins from the nucleus to the cytoplasm where translation occurs. As the infection proceeds, longer unspliced mRNA molecules are produced and translated into multiple proteins from across the proviral genome [83]. Transcribed full-length viral RNA is transported from the nucleus to the cytoplasm for Gag (and Gag-Pol) synthesis, the major structural polyprotein of all retroviruses which comprises four essential subdomains: the matrix, capsid, nucleocapsid and p6. In parallel, singly-spliced RNA is exported and translated to produce viral envelope glycoproteins, initially gp160 which is cleaved into gp120 and gp41 subunits. Both Gag and Env proteins are trafficked to the plasma membrane for the final stages of the viral lifecycle. There are currently no licensed ARV drugs targeting transcription and translation within the HIV life cycle.

1.2.3.5 Assembly and budding

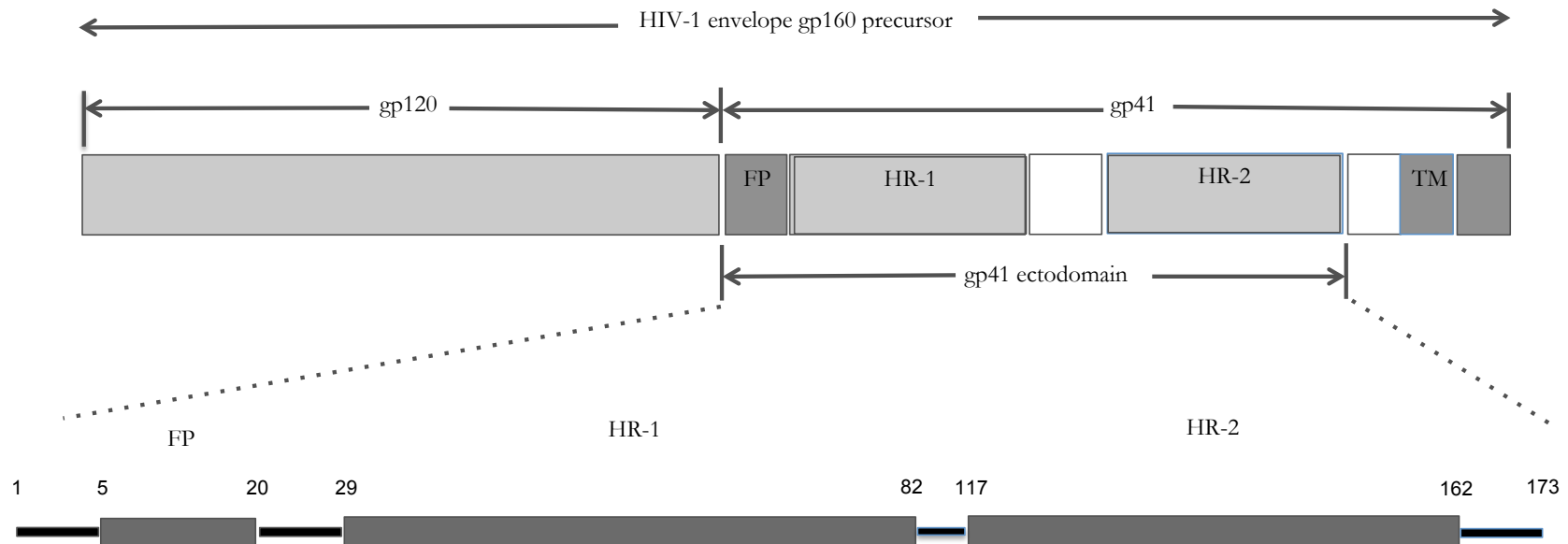
The final stage of the HIV life cycle, virion morphogenesis, is divided into three substages: 1) assembly, where the nascent viral particle is assembled and packaged 2) budding, where the virion obtains its lipid bilayer envelope and finally 3) maturation, where the core of the viral particle condenses to become an infectious particle. Viral assembly is modulated by the subunits of Gag. Following translation, Gag binds via its nucleoprotein subunit to dimeric viral RNA to form a ribonucleoprotein complex. The process occurs within specialised microdomains at the plasma membrane; assembly results in the packaging of all components to create a functional infectious independent virion including two copies of the positive-sense viral RNA, envelope glycoproteins, the Gag polyproteins and the three viral enzymes: integrase, protease and reverse transcriptase [84]. During particle assembly or soon after, protease cleaves Gag into its constituent subunits. This protease-mediated cleavage then results in rearrangement of proteins to produce a dense conical core capsid which is characteristic of the HIV virion. Two classes of ARV drugs, PIs and maturation inhibitors (MIs), target this final phase in the HIV life cycle. While PIs act as competitive inhibitors for the active site of the enzyme to prevent cleavage of Gag, maturation inhibitors act by inhibition of cleavage between protein p24 and a spacer protein in the Gag protein, thus blocking the formation of a mature virion.

1.3 The HIV Envelope as a target for antiretroviral therapy

1.3.1 HIV envelope proteins

Inhibition of viral entry represents a major target for the development of novel ARV drugs. As described, the process of HIV viral entry into cells involves a series of interactions between viral envelope glycoproteins and human cellular receptors. The HIV envelope glycoproteins are derived from gp160, a parental polyprotein encoded within the *env* gene of the HIV genome (**Figure 1.3.1**) [57]. Proteolytic cleavage of gp160 gives rise to two envelope proteins, gp41 and gp120. The proteins are arranged as a trimeric complex of three gp120 subunits which are embedded into the viral envelope and which are non-covalently bound to three gp41 subunits; gp41 subunits extend across the transmembrane domain and stabilise the trimeric complex within the viral lipid bilayer.

Figure 1.3.1: HIV envelope protein, gp160



The schematic shows gp120 and gp41 subunits with a close up view of the gp41 ectodomain; indicated are the subdomains within gp41. FP, fusion peptide; HR-1, heptad repeat 1; HR-2, heptad repeat 2; TM, transmembrane region. The HR-1 and HR-2 subdomains are conjugated via a disulfide bonded-loop. Adapted from: *Lu L. Biochim Biophys Acta. 2012 Dec;1818(12):2950-7*

1.3.2 The three dimensional structure of the envelope proteins

Envelope proteins are arranged as a trimeric complex of three gp120 subunits which protrude from the viral surface and which are non-covalently bound to three gp41 subunits. Together the subunits of this complex serve both to anchor the virus to the target cell and mediate the process of viral entry [57]. The early stages of HIV viral entry have been visualized by crystallographic techniques of the cleaved gp41-gp120 trimer and seem to show similarity to mechanisms of viral entry demonstrated by other viruses [85-89]. Specifically, comparisons have been made with influenza viruses where the envelope proteins, haemagglutinin A1 and A2, fold as non-helical loops in their non-activated state (non-fusogenic), but convert to coiled-coils (fusogenic state) under certain pH conditions [90, 91]. This “loop to coiled-coil” transition forms the basis of a spring-loaded mechanism in which a FP attached to the haemagglutinins is released and pierces target cell membranes [91].

In the case of HIV, after release of FP into the target cell membrane, a pre-hairpin shaped fusogenic state is formed during which α -helical coils of the HR-1 fold into one another to form a coiled-coil [74]. Simultaneously, the three HR-2 domains pack together in an antiparallel manner to form a highly conserved bundle with deep hydrophobic grooves on the surface of the coiled-coil [74]. After piercing of the FP through the cellular lipid bilayer, cellular and viral membranes are pulled into apposition as the HR-1 and HR-2 coiled-coil domains then fold on top of one another with HR-2 aligning into the hydrophobic grooves on HR-1. This so-called “leucine zipper-like” mechanism, a common three-dimensional structural motif found within the secondary structure of proteins (**Figure 1.3.2**), results in mixing of the cellular and viral lipid bilayers as the cell and virus are pulled closer together and effects the creation of a fusion pore through which the viral capsid is released. As this process proceeds, stability of HR-1 and HR-2 hairpin interaction is maintained predominantly through the hydrophobic residues on the HR-1 coiled-coil. Deep hydrophobic grooves termed the “pocket domain” (PD) form on the surface of the coiled-coil which allow the outer residues of the HR-2 helices to align along complementary regions termed the “pocket binding domain” (PBD). The leucine zipper mechanism between the PD and PBD is characterized by a heptad of amino acids beginning with a leucine residue followed by six other amino acids. This is repeated several times and creates a highly thermostable intermediate (**Figure 1.3.2 c**).

Figure 1.3.2: Three dimension representations of the HIV envelope protein, gp41



a. Three dimensional side view representation of gp41 as hairpin bundle structure (created using PyMOL, protein databank sequence 1IF3) b. Three dimensional "overhead" view of gp41 bundle structure (created using PyMOL, protein databank sequence 3F4Y) c. "Overhead view", or helical wheel diagram, of a leucine zipper, with leucine, arranged with other amino acids on two parallel alpha helices. The leucine zipper is characterized by a heptad of amino acids consisting of a leucine residue followed by six other amino acids which is repeated several times. Hydrophobic amino acids are found at the first and fourth residues throughout the region, characteristic of coiled-coils. Adapted from: *Horne WS, Proc Natl Acad Sci U S A. 2009 Sep 1;106(35):14751-6*

1.3.3 HIV fusion inhibitors

FIs are peptides derived from the HIV envelope protein, gp41, which by binding to the pre-hairpin intermediate along the virus-cell fusion pathway, display potent ARV activity. Through molecular mimicry, peptides derived from both the HR-1 and HR-2 domains of gp41 can bind competitively along hydrophobic grooves of the pre-hairpin intermediate coiled-coil bundles of HR-2 and HR-1, respectively. This prevents the formation of the 6-helical hairpin bundle and, thus, interrupts the process of viral-cellular fusion. In addition, viral inhibitory peptides have been described which can target the FP of HIV-1 gp41 and block entry into cells by preventing insertion of the FP into the target cell membrane [69].

1.3.3.1 Anchoring inhibitors

Viral inhibitory peptide (VIRIP) is a naturally occurring peptide first described in 2007; it was identified following screening of a peptide library derived from pooled human haemofiltrate and has since been shown to be derived from the breakdown of α 1-antitrypsin by metalloproteinases [92]. The FP of gp41 is well conserved and intolerant to alterations in its structure. A number of characteristics of VIRIP make it desirable for further investigation as an ARV drug, namely difficulty in selecting resistant HIV variants in *in vitro* culture, novel mechanism of action, and lack of toxicity even at very high concentrations [93]. A purified 20 amino acid derivative of VIRIP, VIR-576, exerts antiviral activity through binding to the hydrophobic gp41 FP, resulting in a conformational change and rapid loss of peptide function. In a 10-day monotherapy study, VIR-576 delivered as an intravenous infusion to 18 patients with HIV, was well tolerated at 5 grams per day and caused a mean drop of 1.23 log₁₀ in HIV RNA. All patients had a HIV RNA of >5000 copies/ml at baseline [93].

1.3.3.2 HR-1-based fusion inhibitors

N51, a 51-residue peptide, was the first described HR-1 (N-HR)-based FI derived from a recombinant protein fragment of gp41 [74]. The N51 peptide associates with C43, a complementary peptide within the HR-2 (C-HR) domain. Crystallographic and circular dichroism studies demonstrated the formation of the classical coiled-coil structure when in association with one another in solution [74]. Although both N51 and C43 have potent antiviral activity, peptides from HR-1 appear to have lower activity than those derived from the HR-2 domain [74]. Indeed, C43 exhibits 10-fold greater antiviral potency that

N51 [94]. A shorter 36-residue peptide (N36; DP-107) corresponding to the central region of N51 was similarly shown to align along HR-2 bundles [95, 96]. In physiological solutions, however, N36, N51 and other similar HR-1-based fusion inhibitor peptides form aggregates such that their overall antiviral potency is diminished [94]. In addition, due to a lack of hydrophilic properties, adaptation of these agents for clinical use has been hampered. Consequently, they have not progressed into clinical trials and their utility has predominantly been for studying HIV viral entry.

1.3.3.3 HR-2-based fusion inhibitors

HR-2-based FIs with antiretroviral activity in the nanomolar range were first described in the early 1990s, namely SJ-2176 [97], DP-178 (known as T-20 and referred to as enfuvirtide hereafter) [98] and C34 [99]. Previous trials of first generation HR-2-based FIs have shown low systemic toxicity profiles, rapid decline in HIV RNA and a low propensity for DDIs. Owing to a lack of oral bioavailability and the availability of newer classes of ARV drugs (notably InSTIs), however, only a small number of agents have progressed into clinical trials. Nevertheless, biophysical properties suggest malleability of HR-2-derived lead compounds. This enables stabilisation of the peptide secondary structure and derivitisation with molecules which can potentiate the circulatory half-life, so renewed interest has focused on their development as LAI ARV drugs.

1.3.3.3.1 Enfuvirtide

The synthetic peptide, enfuvirtide, was the first peptide derived from HR-2 and the only peptide, thus far, to extend beyond phase III studies and into clinical use. This 36-amino acid peptide is a homologue of the HR-2 domain, corresponding to residues 117-162 of gp41 (residues 643-678 of HIV_{LAI}).

Initial *in vitro* activity suggested a half maximal inhibitory concentration (IC₅₀) of around 1.7 ng/mL (0.38 nM) and a level of inhibition approximately 2 Log₁₀ lower than that seen for the HR-1 analogue, DP-125 [96]. Specifically DP125 has an IC₅₀ of 200 ng/mL (44nM). Hence, enfuvirtide was considered the most promising peptide to progress into clinical trials.

In a proof of concept phase I monotherapy study, enfuvirtide was administered intravenously to 16 HIV-positive patients across four dose intervals (3 mg, 10 mg, 30 mg

and 100 mg) twice daily. Following 14 days, significant dose-related declines in plasma HIV RNA were observed in patients who received doses of 30 mg and 100 mg twice daily with a median decline of 1.96 Log₁₀ seen in those receiving 100 mg twice daily. Indeed, within all 4 subjects who received 100 mg twice, HIV RNA declined to below 500 copies/mL, but remained above the threshold of undetectability of 50 copies/mL. Notably, however, median baseline HIV RNA in patients receiving 100 mg twice daily was only 3.83 Log₁₀ (range 3.63 – 4.82 Log₁₀), equivalent to around 4000–60,000 copies/mL, and consequently must be interpreted with caution within this context. Notwithstanding, short-term administration appeared safe and no significant toxicities were observed even at higher doses twice daily over 14 days [100].

Enfuvirtide progressed into a phase II study involving 78 HIV-infected patients with extensive treatment experience to explore the safety, pharmacokinetic profile and antiviral activity when delivered subcutaneously by two modes (self-administered subcutaneous injections vs. a continuous subcutaneous infusion). While injection site reactions (ISRs) were found to be common, these were mild. By subcutaneous delivery, enfuvirtide continued to show dose-related reductions in HIV RNA up to a maximum mean of 1.6 Log₁₀ copies/mL when delivered at a dose of 100 mg twice daily [101]. Potent viral suppression observed in both ARV naïve and experienced participants in phase I and II trials was confirmed in the pivotal phase III TORO 1 and 2 trials. In these, highly treatment experienced patients were given enfuvirtide combined with optimized background therapy (OBT), leading to 1.7 Log₁₀ copies/mL drop in viral load vs. 0.7 Log₁₀ copies/mL drop with OBT alone [78, 100]. ISRs were observed across both studies at a rate of 98%, most of which occurred within the first week of administration but leading to discontinuation in 3%; this, however, has contrasted with post-clinical trial observations where around 80% of patients discontinue by 6 months due to ISRs. Of note, within TORO 1 and 2, pneumonias were observed more frequently in patients treated with enfuvirtide (6.7 vs. 0.6 events per 100 patient-years). However occurrence was within the expected range for this population and possibly related to an immune reconstitution phenomenon in these individuals with advanced immunosuppression and limited treatment options [102]. Moreover, owing to degradation via serum proteinases and peptidases, few DDIs were observed. Only one idiosyncratic reaction was reported between enfuvirtide and the rarely used PI, tipranavir [103]. Thus, enfuvirtide marked a milestone in expanding treatment for patients with few or no treatment options. Notwithstanding a low systemic toxicity profile,

a general lack of DDIs and satisfactory viral kinetic profile however, its short half-life (~ 4 hours) and poor bioavailability has rendered enfuvirtide unpopular with patients [101]. As a result, since its approval in 2003, clinical use of enfuvirtide has declined markedly.

1.3.3.3.2 Optimisation of HR-2-based fusion inhibitors

Primary structure

Following on from the success of enfuvirtide in the TORO 1 and 2 studies [78, 79], various attempts were made to enhance antiviral potency by incorporating regions of the HR-2 subdomain into the structure of new therapeutic peptides. The second generation HR-2-based FI, T1249, has a slightly longer primary amino acid sequence of 39 residues and showed enhanced antiviral potency *in vitro* and was subsequently entered into clinical trials. These same studies demonstrated that with modification of the primary structure, T1249 maintained a higher degree of α -helicity in solution than enfuvirtide, a characteristic which may explain the enhanced potency and extended serum half-life compared with enfuvirtide [104]. However, studies of the crystalline structure of gp41 suggest that, during the process of viral-cell fusion, stability of the 6-helical hairpin bundle is maintained through an interaction between the PD of HR-1 and the PBD of HR-2, rendering the complex highly thermostable. Hence, it has been hypothesized that it is the precise region of HR-2 which is incorporated into design of HR-2-based FIs which is critical to potency, and not the length of the peptide [105]. By incorporating the PBD into the primary amino acid sequence of HR-2-based peptides, it has, therefore, been proposed that the antiviral activity may be accentuated. *In vitro* studies have compared the antiviral potency of rationally designed HR-2-based peptides containing an identical central HR-1 binding domain variably extended to incorporate the PBD, the lipid binding domain (LBD), both the PBD and LBD and with no extended sequences. The inhibitory activity of the HR-2-based peptides containing the central HR-1 binding domain and the LBD or the PBD was improved about 2- and 6-fold, respectively, compared to those containing only the central HR-1 binding domain. However, strikingly, the central HR-1 binding domain plus both LBD and PBD was almost 33-fold more potent than HR-2 peptides containing neither the PBD or LBD [105]. Indeed, small molecule peptides containing only the PBD have been trialled *in vitro* to determine their antiviral potency through destabilising the 6-helical hairpin bundle at the level of the PD alone and show significant promise [106-108]. In keeping with this hypothesis, as a result of only incorporating the LBD, these results are likely to explain the relatively reduced affinity of enfuvirtide to the HR-1 coiled-coil

compared with other experimental HR-2-based FIs and are likely to explain why C34 (derived from amino acid residues 117-150 and overlapping the PBD of HR-2) shows enhanced antiviral potency over enfuvirtide.

Secondary structure

Molecular dynamic studies of enfuvirtide suggest that, when in aqueous solution, the peptide adopts an unstable secondary structure and indeed, several random coiled conformations have been described. Although subcutaneous injection of enfuvirtide avoids digestion by gastric peptidases, it has been hypothesised that through adopting these random coil secondary structures, enfuvirtide becomes vulnerable to rapid degradation by serum proteinases and peptidases [109]. Hence, serum half-life is reduced, resulting in the need for twice daily injections. Furthermore, resulting from a lack of secondary structure in aqueous solutions, the antiviral potency may be diminished owing to reduced affinity of the random coil structure to the coiled-coil of HR-1. Consequently, third generation HR-2-based FIs have been generated in an attempt to improve the secondary structure of peptides targeting the HR-1 coiled-coil complex. By introducing ion pair interactions or “salt bridges” and diaminoalkane stabilizers, the stability of the secondary helical structures, antiviral potency and circulatory half-life of HR-2-based FI peptides has been markedly improved [110, 111]. With these modifications, T2635 (a third generation HR-2-based FI) has superior antiviral potency to enfuvirtide and T1249 in *in vitro* studies, coupled with a higher genetic barrier to resistance [112]. In addition, through full incorporation of the PBD into its primary amino acid sequence, T2635 has *in vitro* activity in the presence of multiple enfuvirtide and T1249-associated mutations.

This dual approach of optimizing both the primary and secondary structure of peptides has been used to develop Sifuvirtide (SFT), an electrostatically constrained peptide. SFT is the only third generation HR-2-based FI to reach clinical trial [113]. Based on structural data of the HIV gp41 core domain and using computer modelling, SFT is a 36 amino acid peptide differing by 9 residues from the parental sequence (and different from C34 in 16 residues) with multiple pairs of basic and acidic residues (glutamic acid and arginine) to promote the salt bridge formation [114]. In addition, SFT was rationally designed to incorporate the PBD. Due to the incorporation of aromatic amino acid into its sequence, the behaviour of SFT has been examined in aqueous media and in relation to its interaction with systems mimicking lipid membranes. Although results showed no significant

interaction of the peptide with cholesterol-rich membranes, greater affinity was noted in lipid membrane models towards a more rigid structure where most of the receptors are found and also to viral membrane suggesting that SFT may accumulate locally at the site of viral-cell fusion [115].

CP32M, a 32 amino acid peptide incorporating sequence immediately adjacent to the PBD is a further example of an electrostatically constrained HR-2-based FI. It is potent within the picomolar range, but remains active in the nanomolar range against a panel of laboratory HIV isolates resistant to both enfuvirtide and C34 [116].

Conjugation of HR-2-based fusion inhibitors

Chemical modification by means of pegylation, linkage of pentasaccharides and cholesterol tagging are ways of optimising the pharmacokinetic profile of multiple therapeutic peptides to extend half-life and improve solubility.

Pegylation is the extension of both peptide and non-peptide molecules with polyethylene glycol (PEG); pegylation can endow molecules with prolonged residence in the body by reducing enzymatic degradation of molecules and reducing the rate at which a compound is eliminated [117]. In addition, PEG molecules have low immunogenicity, are non-antigenic and enhance aqueous solubility [117]. Indeed, attempts to modify enfuvirtide by conjugation to a pentasaccharide linked between molecules via PEG molecules were successful; this conjugated compound showed continued antiretroviral activity within the nanomolar range and when tested in rodent models, had an extended half-life of over 10 hours, compared with 2 hours with the native enfuvirtide molecule. Extrapolating to man, it has been claimed that such a modification may extend the half-life from around 2 hours to 120 hours following subcutaneous injection [118]. Conjugation of both C34 and enfuvirtide to albumin prolonged the half-life in both rodents and human studies [109].

M-T hook

Development of short peptide fusion inhibitors with potency against HIV have mostly involved the C34 lead compound with modifications, such as truncating the compound. Although optimising the primary structure by incorporating the PBD into the molecule has been partially successful, modification of two residues (methionine and threonine) upstream of the native PBD sequence, and the incorporation of these into the sequence

of HR-2-based FI prototypes, has vastly improved potency. For example, compared with the native C34 lead compound, MT-C34 is 6.6-fold more potent than native C34 [119]. Similarly, MT-SFT forms an enhanced thermostable association with the N36 peptide in crystallographic studies relative to SFT. The mechanism is thought to be due to a stabilising effect of the so-called “M-T hook” upon the interaction between PBD and target PD of the 6-HB [120] and is promising for the development of more potent HR-2-based FIs which could be given at lower doses to avoid local injection site reactions.

1.3.3.3.3 Investigational HR-2-based fusion inhibitors

T1249

T1249 is a prototype second generation HR-2-based FI composed of a 39-amino acid consensus sequence derived from HIV-1, HIV-2 and SIV. Although there is complete sequence overlap with enfuvirtide and only partial overlap with the PBD of HR-2, *in vitro* analysis shows enhanced potency over enfuvirtide and ARV activity against HIV isolates with reduced susceptibility to enfuvirtide [121]. An initial phase 1/2 fourteen-day open-label dose-escalation monotherapy study in which 115 extensively treatment-experienced patients received at least one dose of T1249 daily at 192 mg/day showed a dose-dependent decrease in the viral load up to 1.96 Log₁₀ copies/mL [121]. This decrease in HIV RNA is similar to that obtained with the most potent HIV inhibitors [122]. A follow-up phase 2 study conducted in 53 HIV infected adults with detectable viraemia but failing treatment with enfuvirtide showed a median reduction in HIV RNA from baseline to day 11 of 1.26 Log₁₀ copies/mL when enfuvirtide was replaced with T1249 at a dose of 192 mg once daily. These results were despite the presence of mutations within HR-1 inducing reduced susceptibility to enfuvirtide of around 116-fold [123]. Despite the promise of a once daily HR-2-based FI with improved antiviral potency over enfuvirtide and remaining active in the presence of resistance to enfuvirtide, clinical evaluation was not pursued due to problems with drug formulation .

Sifuvirtide

SFT is the only third generation fusion inhibitor which has entered into clinical trials. *In vitro* analysis of a panel of laboratory isolates showed SFT to have enhanced potency over enfuvirtide of up to 21-fold for some laboratory strains [124]. A phase 1a study in 60 healthy volunteers found SFT to be safe and well tolerated with, only mild injection-site reactions when delivered subcutaneously. Pharmacokinetic studies of single and multiple doses showed half-lives were 20.0 ± 8.6 hours and 26.0 ± 7.9 hours, respectively, equating

to about 6 times longer than those observed for enfuvirtide [124]. When trialled in HIV-positive patients over a 28 day period at doses of 10 mg and 20 mg once daily, the half-life of SFT was 17.8 ± 3.7 hours for 10 mg group and 39.0 ± 3.5 hours for 20 mg group. This was up to 10 times longer than enfuvirtide and suggests that twice-weekly dosing may be feasible [113]. It has been proposed by the investigators that the improved pharmacodynamic profile observed with SFT *in vitro* compared with enfuvirtide may be as a result of three factors, namely 1) incorporation of the PBD 2) enhanced affinity towards lipid membranes on the viral surface 3) an improved pharmacokinetic profile [113]. ISRs occurred at a rate of 7% throughout trials, contrasting significantly with a rate of 98% with enfuvirtide in the TORO 1 and 2 trials. Pharmacodynamics results have not been reported for SFT as yet. Finally, in addition to an extended half-life compared to enfuvirtide, SFT was shown to be absorbed more rapidly with a time to maximum concentration ($T_{\max}$) observed only 3 hours post-dosing compared with $T_{\max}$ 6-7 hours in trials with enfuvirtide [113, 125].

Albuvirtide

Albuvirtide (ABT) is a prototype conjugated first generation HR-2 FI. It is a derivative of residues 117 to 151 of HR-2, modified by three amino acids to improve solubility and by the addition of a long-chain fatty acid, 3-maleimidopropionic acid (MPA) to the thirteenth residue of C34 (substitutes serine to lysine) as a side chain. The peptide forms irreversible covalent bonds with serum albumin in a molecular ratio of 1:1. Through addition of MPA, the circulatory half-life of was extended from 1.67 hours to 25.8 hours when injected intravenously in rats and from 10.89 hours to 104.2 hours in non-human primates. In a proof-of-concept study in which treatment-naïve patients with HIV RNA >5000 copies/mL were enrolled in a randomised placebo-controlled single-dose escalation study and open-label multiple dose of either 160 mg or 320 mg of intravenous ABT, it showed the drug to be well tolerated. The plasma HIV RNA remained suppressed for up to 10 days following a single injection. Patients exhibited a mean decline of 0.68 Log₁₀ copies/mL following a single dose of 160 mg but 1.05 Log₁₀ with a dose of 320 mg. In addition, ABT showed a plasma half-life of around 11.5 days. In a Phase 3 open label study, ABT with lopinavir/ritonavir was evaluated at two doses (160mg and 320 mg weekly) in twenty ART naïve patients, over 7 weeks. Although a decline of decline of 1.9 Log/ml and 2.2 Log/ml was observed with the dose 160 mg and 320 mg respectively, only 11.1% and 55.6% of patient achieved HIV RNA < 50 copies/ml at week 7 [126]. More recently, a randomized phase 3 licensing study reported a head-to-head comparison of weekly ABT plus twice-

daily lopinavir/ritonavir with lopinavir/ritonavir plus a dual NRTI backbone (lamivudine with either tenofovir, abacavir or zidovudine). In 389 treatment-experienced patients experiencing virological failure, 80.4% of the ABT group attained a HIV RNA < 50 copies/ml by intent-to-treat analysis by week 48, compared to 66% in the triple-drug group. Moreover, ABT was well-tolerated with mild diarrhoea reported in around 7.5% compared with 14% in the triple therapy group, a side effect most likely related to lopinavir/ritonavir [127].

Table 1.3.1 Subclasses of HIV entry inhibitors entered into clinical trials

Viral entry step	Drug class	Mode of action	Drug	Phase of development	Reference
gp120-CD4 binding	gp120 attachment inhibitors	Binds to region within CD4 binding pocket of gp120 and prevents interaction between CD4 and gp120.	BMS-488043	Discontinued due to suboptimal pharmacokinetic profile.	Hanna <i>et al</i> , 2011 [128]
			BMS-663608 (Fostemavir)	Phase 2a study: 1.7 log ₁₀ reduction in HIV RNA; phase 2b study of BMS-663068 plus RAL/TDF versus RAL/TDF/ritonavir-boosted ATZ completed.	Lalezari <i>et al</i> , 2015 [129]
	gp120 post-attachment inhibitors	Anti-CD4 humanised monoclonal antibody: after gp120 attaches to CD4 receptors, ibalizumab binds to a conformational epitope (domain 2 of extracellular CD4) on CD4 and prevents interaction of gp120 with CCR5 or CXCR4.	Ibalizumab	Phase 2b: 24-weeks of therapy, dosed every 2 weeks in combination with optimized background regimen (OBR) - 44% of individuals achieved HIV RNA below level of quantification.	Henrich <i>et al</i> , 2013 [130]

gp120-coreceptor binding	CCR5 antagonists	Negative allosteric modulator of CCR5; binds to CCR5, thereby blocking the HIV protein gp120 from associating with the receptor. *Also binds CCR2: influence on HIV unclear.	Maraviroc	FDA approval since 2007 post Phase 3 studies (MOTIVATE 1 and 2): double-blind, placebo-controlled, phase 3 studies-Maraviroc versus Optimized Therapy in Viraemic Antiretroviral Treatment-Experienced Patients in patients with R5 tropic HIV.	Gulick RM <i>et al</i> , 2008 [77]
			Cenicriviroc*	Phase 2b: 48 week TDF/FTC/Cenicriviroc(CVC) 100mg vs. TDF/FTC/CVC 200mg vs. TDF/FTC/EFZ: 68%, 64%, 50% HIV RNA <50 copies/mL by week 48. Phase 3 commenced.	Thompson M <i>et al</i> , 2016 [131]
			Aplaviroc	Development terminated due to idiosyncratic drug-induced hepatitis and inferiority as third agent compared to efavirenz in phase 2b ASCENT trial.	Nichols WG <i>et al</i> , 2008 [132]
			Vicriviroc	Development terminated after Phase 3 Vicriviroc plus OBR vs Placebo plus OBR failed to demonstrate virological superiority.	Caseiro MM <i>et al</i> , 2012[133]
	CCR5 antibody	Humanised IgG4K monoclonal antibody that binds CCR5 and inhibits R5-tropic HIV	PRO-140	Phase 2b/3 two-part study: will evaluate safety, efficacy and tolerability of PRO-140 delivered by subcutaneous injection weekly and in conjunction with existing cART. After 1 week, all participants will enter a single arm open label extension. Previously, a single subcutaneous injection effected a HIV RNA reduction of 2.5 Lg following a single dose.	Tenorio A <i>et al</i> , 2011 [134]

				Phase 2b/3 two-part study (NCT02483078), will evaluate the efficacy, safety, and tolerability of PRO-140 in conjunction with existing antiretroviral therapy (ART) (failing regimen). In this study, PRO140 (350 mg) is administered weekly SC with existing ART for one week. After one week, all subjects enter the 24-week single-arm, open-label treatment period	
	CXCR4 antagonists	<i>In vitro</i> potent activity against X4 tropic HIV; blockade of CXCR4 coreceptor to prevent HIV entry into susceptible cells.	AMD3100	Phase 2 study involving 40 HIV positive individuals either stable on ART or not on ART with HIV RNA >5000 copies/mL. Discontinued after no pharmacodynamic effect found in vivo.	Hendrix CW <i>et al</i> , 2000 [135]
gp41 mediated cell fusion	HR-1-based fusion inhibitors	Molecular mimics of HR-1: bind to hydrophobic grooves of HR-2 coiled-coil of gp41 to block formation of the 6-helical hairpin bundle	-	No compounds developed for clinical trials; hydrophobicity limits development as agents.	-

HR-2-based fusion inhibitors	Molecular mimics of HR-2: <i>in vitro</i> bind to deep hydrophobic grooves of HR-1 coiled-coil of gp41 to block formation of the 6-helical hairpin bundle	Enfuvirtide	FDA approved since 2003 post Phase 3 studies (TORO 1 and 2): addition of enfuvirtide to OBR vs OBR alone provided additional 0.77 log ₁₀ copies/mL reduction in HIV RNA in highly treatment experienced patients.	Lazzarin A <i>et al</i> , 2003 [79] Lalezari JP <i>et al</i> [78]
		T1249	Phase 2: once or twice daily at 192 mg/day caused a dose-dependent decrease in the viral load up to 1.96 log ₁₀ copies/mL. Discontinued after Phase 2 due to formulation difficulties.	Eron JJ <i>et al</i> , 2004 [121]
		Sifuvirtide	Phase 1a: 60 healthy volunteers safe well tolerate. Phase 2b pharmacokinetic study suggest twice weekly dosing possible; 7% injection site reactions. No pharmacodynamics results published. Future planned studies unclear.	He Y <i>et al</i> , 2008 [124] Meng Q <i>et al</i> , 2014 [113]
		Albuvirtide	Phase 2: 1.05 Log ₁₀ with a dose of 320 mg monotherapy; plasma half-life of around 11.5 days. Phase 3 head-to-head studies completed, as above (ABT in combination with lopinavir/ritonavir vs. tenofovir/emtricitabine/lopinavir/ritonavir)	Saag MS <i>et al</i> , 2012 [136] Zhang H <i>et al</i> , 2016 [126]
Fusion peptide inhibitors (Anchoring inhibitors)	Interacts with the FP of gp41 preventing the peptide from penetrating into target cell	VIR-576	Phase 1: 10-day monotherapy trial with treatment-naïve, HIV-1-infected individuals with viral loads of ≥10,000 copies per ml; intravenous infusion of VIR-576 reduced the mean plasma	Forssmann WG <i>et al</i> , 2010 [93]

		membranes and begin the mechanism of pulling the		viral load by 1.23 Log ₁₀ copies per ml without causing severe adverse effects.	
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C34-PEG₄-Chol

It could be argued that up until now conjugated first generation HR-2-based FIs, as well as second- and third-generation compounds, have failed to overcome significantly the shortcomings of enfuvirtide. Despite clinical trials demonstrating the half-lives of T1249, SFT and ABT to be longer than enfuvirtide and notwithstanding robust safety data, potent antiviral activity and persistent activity in the presence of some enfuvirtide-associated resistance mutations, the acceptability of an ARV drug which requires injection more than once a week is questionable.

C34 is a lead compound peptide made up of 34 amino acids derived from the HR-2 domain (C-terminal) of gp41. Its sequence is homologous to residues 117-151. This is in contrast to enfuvirtide whose sequence extends from residue 127-162 of HR-2. It encompasses the PBD of HR-2 and indeed, *in vitro*, C34 exhibits approximately a 50-fold increased fusion inhibition over enfuvirtide, which is hypothesized to be due to the inclusion of the PBD [19]. Although discovered around the same time as enfuvirtide, the hydrophobic character of the molecule meant that C34 was less amenable to development as an ARV drug. The main use of C34 until now has been as a research tool to aid in the discovery of the complete structure of gp41. Like enfuvirtide and being a peptide, it is unlikely to have oral bioavailability due to digestion by gastric enzymes.

Several critical steps in the HIV life cycle, including viral entry, assembly and budding, take place in specialised plasma membrane microdomains. In particular, a growing body of evidence shows that cholesterol- and sphingolipid- enriched microdomains known as lipid rafts [23, 24] play a critical role in multiple steps in the replication cycle of enveloped viruses, including HIV [25, 26], influenza [27, 28], Hepatitis C [29], rhinovirus [30] and herpes virus [31]. For HIV, in particular, convincing evidence of its fusion at, and budding from, lipid rafts comes from the composition of its lipid membrane, which differs from that of the host cell membrane in that it is particularly enriched in cholesterol and sphingomyelin [32]. For example, in HIV membranes there is a 3.2 fold increase in sphingomyelin and a 3.6-fold increase in phosphatidylcholines compared with eukaryotic plasma membranes. Association with lipid rafts is promoted by a highly conserved motif located in the proximal to transmembrane region of gp41 [33, 34]. Moreover, the primary receptor for HIV, CD4, is one of the proteins that is particularly enriched in lipid rafts. In addition, gp41 associates with Caveolin-1 [35], the structural protein component of a

subset of lipid rafts known as caveolae [36]. Since Caveolin-1 is a cholesterol-binding protein [37], cholesterol is enriched in caveolae on the HIV membrane. Thus, targeting an ARV drug to membrane microdomains to increase their affinity toward membrane-bound receptors has long been considered as a means of increasing drug potency. [20-22].

C34-PEG₄-Chol is a novel HR-2-based FI derived from the 34 amino acid sequence stretching across the HR-2 and conjugated to a cholesterol moiety (Chol) via glycine-serine-glycine-cysteine residues and polyethylene glycol molecules (PEG₄) (**Figure 1.10**). In developing the compound, it was reasoned that cholesterol would endow the conjugated peptide sequence with affinity for lipid rafts. The sequence chosen to conjugate to cholesterol was C34, because a) it is more potent *in vitro* than enfuvirtide and b) enfuvirtide already includes a hydrophobic C-terminal segment which drives insertion into lipid membranes [25, 37-40]. Two cholesterol derivatives of C34, C34-Chol and C34-PEG₄-Chol, differing only at the polyethylene glycol spacer (PEG₄), have been produced [137]. By tagging the C34 molecule onto cholesterol moieties, this allows anchoring of the C34 peptide directly to the cellular domain where gp41-mediated fusion occurs. Accordingly, by directing C34 to the site of fusion, *in vitro* analysis has shown antiviral potency to be enhanced by a factor of 80-200-fold over enfuvirtide (**Table 1.3.2**).

Table 1.3.2 Comparison of the antiviral potency of C34-PEG₄-chol with other fusion inhibitors on a panel of HIV isolates.

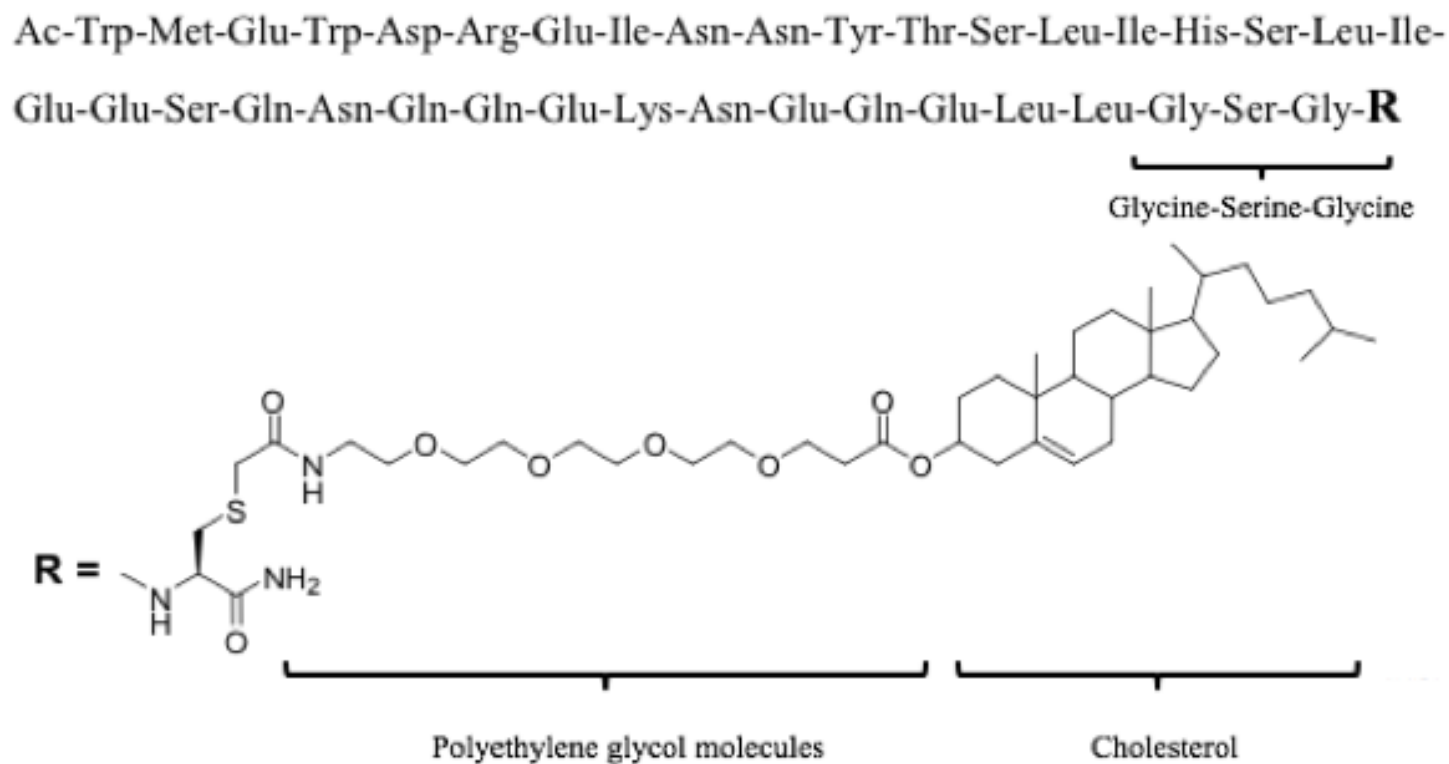
HIV Strain	Subtype isolate	IC ₉₀ Viral Infectivity (nM)				
		C34-PEG ₄ -Chol	ENF	T1249	C34	C34-Chol
DJ258	A (Primary)	0.08	7.6	4.5	4.8	0.20
JFRL	B (Primary)	0.11	20.0	8.3	20.0	0.16
NL4-3	B (TCLA clone)	0.05	6.1	2.6	5.4	0.16
CC7/86	B (Primary)	0.14	32.0	24.0	22.0	1.00
94ZW103	C (Primary)	0.13	11.0	1.9	5.3	0.41
UG270	D (Primary)	0.54	53.0	5.2	51.0	1.50
CM235	CRF01_AE (Primary)	0.60	61.0	11.0	20.0	1.80
BZ162	F (Primary)	0.11	9.2	2.2	6.1	0.37

The IC₉₀ for inhibition of viral infectivity of a number of primary isolates in human PBMCs is shown for C34-PEG₄-chol, enfuvirtide (ENF), the second-generation fusion inhibitor T1249, C34 and C34-chol. Provided through personal communication from: *Dr Antonello Pessi, PeptiPharm, Rome, Italy*

Linking of C34 with cholesterol increases markedly its half-life in mice, relative to C34 alone, by enhancing albumin binding (**Figure 1.3.5**). Furthermore, through addition of a PEG₄ spacer between the cholesterol moiety and the C34 peptide sequence, solubility of the compound in aqueous media is enhanced, allowing up to 20mg to be delivered in 1ml of solute and for it to be developed as a potential ARV for use at a dose which is unlikely to induce toxicity [41].

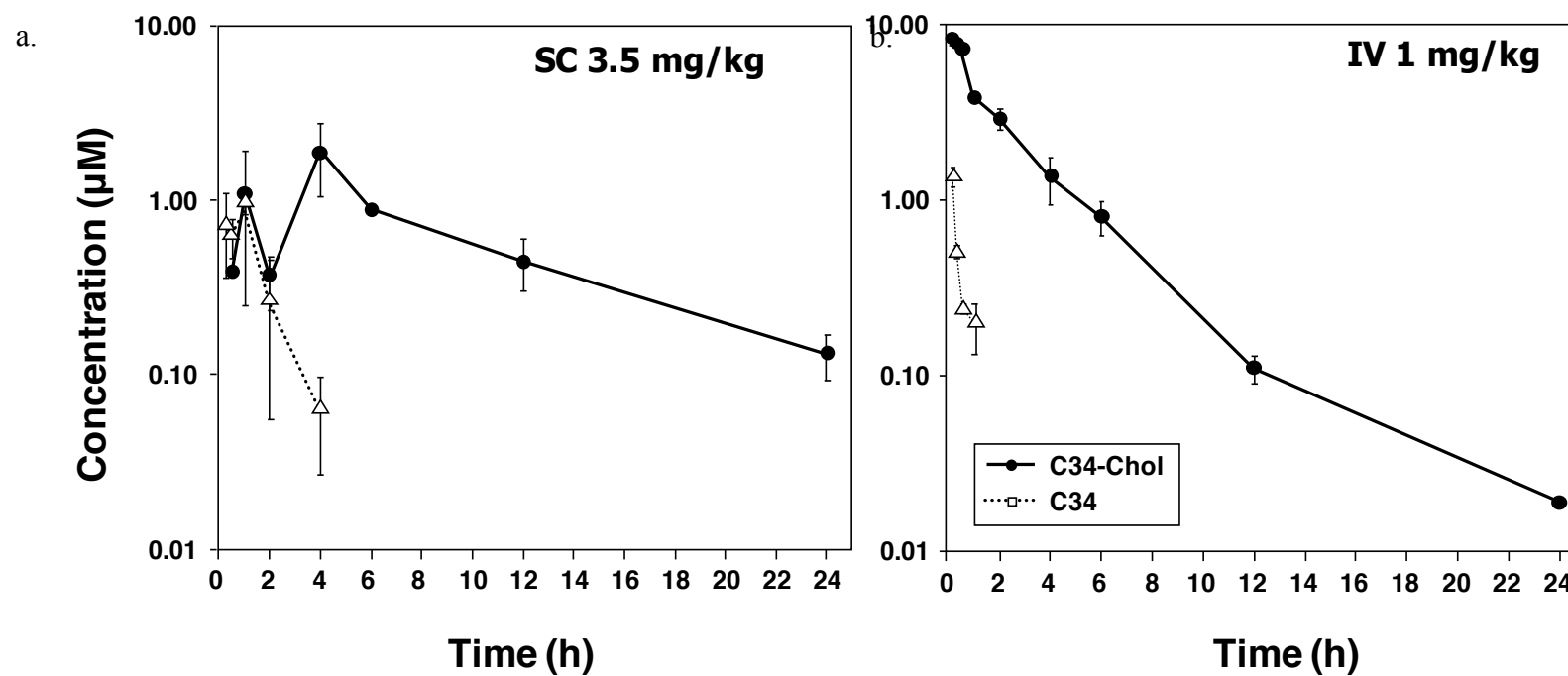
A phase 1 (two-stage) first-in-man clinical trial is planned to assess the safety, pharmacokinetic profile and antiviral efficacy of single dose (stage 1) and then multi-dose delivery (stage 2) of C34-PEG₄-Chol in men chronically infected with HIV, who are not currently receiving ART. Within this trial, selection of an efficacious dose of C34-PEG₄-Chol will be based on *in vitro* antiviral potency and predicted exposure in humans from animal pharmacokinetic data. For complete suppression of viral replication, the target plasma concentration at trough should be $\geq 10 \times IC_{50}$ which, depending on the viral strain tested *in vitro*, ranged between 0.5 and 0.05 nM. The plasma clearance of C34-PEG₄-Chol in man, predicted by allometric scaling of the preclinical pharmacokinetic data (0.06 mL/min/kg), is very similar to the observed clearance in rhesus monkeys. In the latter, the plasma concentration of C34-PEG₄-Chol is ~ 50 nM 72 hours after subcutaneous administration of a 2 mg/kg dose, a value 100-fold higher than the IC_{50} on the more neutralization-resistant viral strains, UG270 and CM235 (**Table 1.3.2**). Dose-proportionality in humans and comparable subcutaneous bioavailability in monkeys and humans is assumed, as is typically observed for peptides. Thus, it has been calculated that a 0.2 mg/kg dose should provide $10 \times IC_{50}$ exposure up to 3 days after administration and that a dose range of 10-80 mg should be explored to identify the most suitable dose for 72 hours (twice weekly) subcutaneous dosing in humans. The starting dose of 10 mg, although unlikely to provide drug coverage for three days, should still be suitable for once-daily administration. It provides a minimal efficacious dose at which no toxicities will arise during drug dose escalation. The starting dose was also arrived at on the basis of the safety profile that emerged from animal studies.

Figure 1.3.4: Chemical structure of C34-PEG4-Chol



Structure of C34-PEG₄-Chol: the 34 amino acid sequence is linked to four polyethylene glycol molecules via glycine-serine-glycine residues. The cholesterol is attached to the polyethylene glycol sequence. The molecular weight of C34-PEG₄-Chol is 5268 Daltons. Provided through personal communication from: *Dr Antonello Pessi, PeptiPharm, Rome, Italy*

Figure 1.3.5: Comparison of plasma half-life of C34 and C34-PEG₄-Chol in mice following subcutaneous and intravenous delivery



Following cholesterol tagging, C34-PEG₄-Chol shows a marked extension in its plasma half-life in mice when compared with the native C34 molecule; results are shown for both (a) subcutaneous (sc) and (b) intravenous (iv) injection. Provided through personal communication from: *Dr Antonello Pessi, PeptiPharm, Rome, Italy*

1.3.3.3.4 Resistance to HR-2-based fusion inhibitors

The presence of drug resistant variants prior to treatment initiation, usually as a consequence of transmitted drug resistance, is an independent predictor of treatment failure in HIV-infected patients [138]. Prospective studies have shown that access to genotypic and phenotypic testing is associated with improved morbidity and mortality outcomes relative to controls [138, 139]. As a result, elucidating the viral genotypes that are resistant to a particular drug and determining the genetic barrier to resistance is critical to the development of new ARV compounds.

As a result of sharing around two-thirds homology, it was assumed that the mode of action of C34 and enfuvirtide is similar. Functional studies and peptide scanning strategies of the interactions of enfuvirtide and C34 peptides with HR-1 derived peptides, however, have raised doubt and suggested their modes of action may differ and depend more crucially on residues lying outside of their zones of homology. Specifically, enfuvirtide attaches to the lipid domain (LD) of HR-1 and to around two-thirds of the central HR-1 binding domain. Conversely, C34 aligns along the central HR-1 binding domain and the PD of HR-1. The HR-2-based FIs engineered to contain neither the LBD nor PBD possess only marginal anti-HIV activity [140]. Moreover, potentially because of the incorporation of the PBD into its sequence and the critical importance of the PD in maintaining stability of the 6-HB, native C34 molecules have better antiviral activity than enfuvirtide, suggesting that the resistance profiles of C34 and enfuvirtide may indeed differ.

The initial *in vitro* studies of enfuvirtide delineated three residues adjacent to the LD of HR-1, namely the “GIV motif” (residues 36-38) as primary resistance sites. Subsequently, *in vivo*, the zone of resistance has been shown to extend between residues 36-45, with various amino acid substitutions capable of endowing resistance to enfuvirtide. Limited *in vitro* studies conducted with the native C34 molecule indicate emergence of mutations at positions 33 and 38 conferring moderate resistance, while other peptides analogous to C34 which interact with the PD have selected various mutations between residues 37 and 79 of HR-1. Notably, however, mutations appear to skip the gp41 PD, suggesting that residues within the sequence are highly conserved. Mutations arbitrarily introduced into the PD via site-directed mutagenesis, however, have rendered viruses highly resistant to native C34 molecules, while remaining sensitive to enfuvirtide [76, 141].

While mutations within the HR-1 region appear to endow viral resistance to HR-2-based FIs, such amino acid substitutions reduce fitness of the virus. These substitutions may destabilize the formation of the 6-HB by disrupting packaging and the electrostatic and hydrophobic interactions between residues [142]. Compensatory mutations occurring within the HR-2 subdomain can improve affinity of the HR-2 subdomain to the HR-1 domain in the presence of primary resistance mutations, further reducing susceptibility to FIs and restoring viral fitness and infectivity.

1.4 Hypotheses

1.4.1 Hypothesis 1: In the presence of residual HIV viral replication, drug pressure will result in the selection of mutations in the HR-1 region of gp41.

It is predicted that the culture of HIV in the presence of suboptimal concentrations of C34-PEG₄-Chol will result in the evolution of drug-resistant virus. On population sequencing, resistance-associated mutations will be identified within the HR-1 domain of gp41.

1.4.3 Hypothesis 2: Mutations occurring in the presence of C34-PEG₄-Chol will confer reduced susceptibility of HIV to the drug.

The HR-2-based FI binding along the deep hydrophobic groove will be impeded by the presence of mutations introduced along the HR-1 domain, allowing viral entry to proceed and thus confer reduced drug susceptibility.

1.4.3 Hypothesis 3: Alternative clinical trial designs will yield more useful clinical data compared to a standard clinical trial design.

Alternative clinical trial designs, utilising accumulating data during the progress of the trial to inform subsequent dose increment decisions, are used commonly in oncology to determine the maximum tolerated dose (MTD) of new chemotherapeutic agents. The higher doses normally equates to an improved clinical response. The aim of alternate designs is to determine the MTD such that the lowest number of patients is exposed to sub-therapeutic doses of a drug. They afford rapid determination of optimal dosage. In addition, more pharmacokinetic and toxicity data are obtained at doses which are likely to be therapeutic and so the dose-response curve may be better evaluated at therapeutic doses. In terms of participant numbers, these trial designs have a major advantage over standard clinical trials where, fixed numbers of participants are exposed to a predefined dose level before incrementing to the next dose level, thus meaning a large number of participants are exposed to sub-therapeutic doses before the optimal dose is reached. To date, these alternative clinical trials have not been used in the field of investigational ARV drugs.

1.4.4 Hypothesis 4: As the plasma concentration of C34-PEG₄-Chol increases, there will be a greater decline in plasma HIV RNA.

As the plasma concentration of C34-PEG₄-Chol increases with higher doses of the drug, a greater reduction in HIV RNA will be seen in plasma.

1.4.5 Objectives

These hypotheses will be tested in the following ways:

1. Resistance mutations likely to be associated with reduced susceptibility to C34-PEG₄-Chol *in vitro* will be determined as follows:
 - a. Laboratory clades of HIV will be cultured with susceptible human cell lines in the presence of sub-limiting concentrations of C34-PEG₄-Chol to exert drug pressure on the viral quasi species and induce selection of drug resistant mutant virus.
 - b. Overlapping oligonucleotide primers will be designed to amplify and sequence the entire length of gp41 by polymerase chain reaction (PCR).
 - c. Sequencing will be undertaken pre- and post- passage of HIV cultures with C34-PEG₄-Chol to allow resistance pathways to be inferred.
2. Resistance mutations selected *in vivo* when participants are exposed to selective drug pressure will be defined by:
 - a. Extracting plasma HIV RNA from patients pre- and post-exposure to C34-PEG₄-Chol.
 - b. Using the overlapping oligonucleotide primers developed for *in vitro* experimentation within the context of the clinical trial to sequence gp41 from patient samples.
3. Change in drug susceptibility induced by mutations found *in vitro* and *in vivo* will be assessed by undertaking site-directed mutagenesis to create a viral clone which can be grown in the presence of drugs with a luciferase reporter cell line to assess change in susceptibility induced by point mutations.
4. Explore and simulate clinical trial designs for stage 1 of the study to find the design which will yield maximum pharmacokinetic data with 28 patients.

Chapter 2

Laboratory materials and methods

2.1 Research approvals

Approvals for experiments was obtained from the Imperial College London Genetic Modification (GM) Committee and from the Health and Safety (HSE) executive. All viral transfections and cultures were carried out within Containment Level (CL) 3 laboratories within the Jefferiss Trust Laboratories, Wright-Fleming Institute on the St. Mary's Campus. RNA extraction, reverse transcription, amplification by nested polymerase chain reaction (PCR) and sequencing were performed in the molecular diagnostic unit (MDU), Wright-Fleming Institute. Finally, site-directed mutagenesis and cloning experiments were performed in the Variety Wind D laboratories, Wright-Fleming Institute. All procedures were conducted according to Imperial College London Codes of Practice.

2.2 Peptide synthesis

C34-PEG₄-Chol was gifted by Dr. A. Pessi (PeptiPharma, Rome, Italy). The peptide was synthesised by solid phase using Fast moc/tBu chemistry on a peptide synthesizer, by chemoselective thioether conjugation between the Cys-C34 precursor (Ac-WMEWDREINNYTSLIHSLEESQNQQEKNEQELLGSGC) and the cholesterol derivative. The compound was purified by reverse-phase high-pressure liquid chromatography (HPLC) and identities verified by mass spectrometry, as described by Ingallinella *et al*, 2009 [143]. The peptide was supplied as a lyophilised powder, stored at -20°C and reconstituted in phosphate-buffered saline (PBS) in stock aliquots of 100 µM concentration and then diluted in cell culture medium to the required concentrations for viral culture experiments.

2.3 Generation of virus stocks

2.3.1 Cell resuscitation, culture and storage

Cell lines listed below (**Table 2.1**) were cultured in a 5% CO₂ humidified atmosphere at 37 °C.

Table 2.1: Cell lines

Cell line	Description	Culture conditions	Reference
PM-1	Clonal derivative of HUT-78 cells. Cells are permissive of macrophage and T-cell tropic viruses.	RPMI 1640, P/S, 10% FCS	Lusso P <i>et al.</i> , 1995
C8166	Human umbilical cord blood lymphocytes. Morphology is T-lymphoid. About 1% of cells contain lobulated nuclei and 5% are multinucleated. This cell line carries but does not express the HTLV-1 genome, which has been used as a target for HIV-1 infection.	RPMI 1640, P/S, 10% FCS	Salahuddin <i>et al.</i> , 1983
C8166-R5	Human umbilical cord blood lymphocytes expressing R5 and X4 co-receptors. Morphologically the same as C8166 cells.	RPMI 1640, P/S, 10% FCS	Krowicka H <i>et al.</i> , 2008
HEK 293T	Primary human embryonic kidney cells transformed by sheared human adenovirus type 5 (Ad 5) DNA. Cells are 3% CD4+, 3% CD20+.	DMEM, P/S, 10% FCS	Graham FL <i>et al.</i> , 1977
TZM-bl	The TZM-bl indicator cell line contains integrated reporter genes for firefly luciferase and E-coli β -galactosidase. When infected with HIV, the LTR activates the reporter genes. Luciferase activity can then be quantified by application of a buffer and measurement of luminescence to quantify the proportion of infected cells.	DMEM, P/S, 10% FCS	Platt <i>et al.</i> , 1998

DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; P/S penicillin (250u/ml) and streptomycin (250 μ g/ml)

Cell lines were obtained from the Centre for AIDS Research (CFAR) at the National Institute for Biologic Standards and Control (NIBSC), Potters Bar, UK.

Resuscitation of cells: Upon receipt, cell lines were kept frozen in a -150 °C freezer within the CL3 laboratories. Prior to cell resuscitation, culture flasks were prepared by labelling with the cell line name, passage number and date. An ampoule containing the frozen cell line was transferred from the -150 °C freezer to the microbiological safety cupboard in a closed polystyrene container. The ampoule containing frozen cells was wiped with paper towel soaked in 70% alcohol around the cap. The cap was turned 1/4 to release any residual liquid nitrogen that may be trapped and held within the palm to gently warm and loosen the frozen cell pellet. Cryoprotectant was separated off from the cells: once loosened from the ampoule, the frozen cell pellet was immediately emptied into a pre-prepared Falcon tube containing 25 mls of the appropriate complete cell culture medium to dilute the cryoprotectant. The Falcon tube was placed in a centrifuge and centrifuged at 150 x g for 5 minutes. The Falcon tube was then transferred back to the microbiological safety cabinet and the cell culture medium was decanted off into virkon solution. The cell pellet was resuspended in 3-5 mls of the appropriate complete cell culture medium by gently pipetting up and down using a sterile 10 ml pipette. Cells were transferred into the pre-labelled culture flask and placed in an incubator under standard conditions overnight. The following morning, a further 3-5 mls of complete cell culture medium was added.

Cell culture: Suspension cell lines were maintained in complete cell culture medium comprised of RPMI 1640 (Sigma Aldrich), 10% fetal calf serum (FCS) heat inactivated at 56°C for 45 minutes (Sigma Aldrich) and 1% penicillin-streptomycin (Sigma Aldrich). Cells were passaged every 3-4 days in 25cm² or 75cm² flasks; complete cell culture medium was monitored daily for change in colour from pink to orange. When colour change was observed, approximately 3/4 of the volume within the flask was removed and replaced with the equivalent volume of new complete cell culture medium. Suspension cell lines were cultured in 5-6 mls or 8-10 mls of complete cell culture medium, depending on the size of the flask.

Adherent cell lines were maintained in complete cell culture medium comprised of Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich), 10% FCS heat inactivated at 56°C for 45 minutes and 1% penicillin-streptomycin. Cells were removed from the base of the flask using trypsin every 3-4 days. Briefly, spent complete cell culture medium was removed from the flask and disposed of in virkon solution. Approximately 3-4 mls of trypsin was pipetted into the flask and swirled gently across the plane of cells to remove cellular debris before removing again and disposing in virkon solution. A further 5 mls of trypsin was then added to the culture flask to cover the film of adherent cells on the base of the flask. After approximately 2-3 minutes, the flask was gently tapped to remove the adherent cells from the base of the culture flask. A further 8 mls of complete cell culture medium was added to the cell/trypsin mix. Approximately 5 mls of the cell/trypsin mix was disposed of within virkon solution and replaced with fresh complete cell culture medium within the flask and passaged in 75cm² flasks with 8-10 mls of complete cell culture medium. The cell culture flask was then placed on its side within the incubator (at 37°C with 5% CO₂).

2.3.2 HIV isolates

BaL and NL4-3 (derived by transfection of the infectious molecular clone pNL4-3 into HEK 293T cells) were used to generate C34-PEG₄-Chol resistant viruses. The R5 clonal virus, BaL, originally cloned by Drs. S. Gartner, M. Popovic and R. Gallo, was obtained from the CFAR at the NIBSC.

The X4 HIV-1 proviral molecular clone, pNL4.3, originally cloned by Dr. M. Martin, was also obtained from CFAR at the NIBSC and NL4-3 stocks were generated by transfection of HEK 293T cells with the proviral DNA, as described below.

2.3.3 Transfection of pNL4-3 into HEK 293T cells

NL4-3 was generated after transfection of the plasmid, pNL4-3, into HEK 293T cells. One day prior to transfection, 1 x 10⁵ HEK 293T cells were plated onto 3 wells of a 6-well plate in the presence of complete DMEM; cells were allowed to grow to 70-90% confluence. On the day of transfection, 100 µl incomplete DMEM "serum-free medium" was mixed with 2 µg of pNL4-3 in a 15ml polystyrene Falcon tube with 12 µl of polyethylenimine (PEI, 1 µg/1 ml) added. Solutions were pulse-vortexed 15 times for 1 second each and incubated for 20 minutes at room temperature. The medium was removed

from the HEK 293T cells and cells washed with PBS, followed by dripping 700 µl of pNL4-3/PEI/DMEM solution to cover the well. After incubation of the cell/transfection mixture for a further 3 hours, 2 ml of complete DMEM were added and incubated for a further 2 days at 37°C, 5% CO₂. After approximately 48 hours, culture supernatants were collected from the wells using a 5 ml pastette; supernatants were centrifuged at 500 x g for 5 minutes and separated into 500 µl aliquots for freezing at - 80°C.

2.3.4 Expansion of virus stock

PM-1 cells were thawed, resuscitated and propagated in complete RPMI 1640. After approximately 1 week, cells were centrifuged and the cell pellet re-suspended at a density of 5×10^5 cells/ml in complete RPMI 1640; 1 ml of each viral supernatant was then allowed to adsorb onto 500 µl of the re-suspended PM-1 cells (total 2.5×10^5 cells) for between 1 and 4 hours at 37 °C, 5% CO₂ in a 6-well plate. After incubation for 1-4 hours, 1 ml of culture medium was removed, replaced with fresh medium and incubated for 72 hours at 37 °C in 5% CO₂. The infected PM-1 cells were then counted and half were transferred to a T-25 cell culture flask; approximately a 4-fold excess of uninfected PM-1 cells was then added to the infected cells and were suspended in 5-6 ml of complete RPMI 1640 medium and incubated upright overnight in a T-25 flask. The following morning, the culture was made up to 18 ml with complete RPMI 1640 and incubated for a further 4 days, after which time, the viral supernatants containing NL4-3 and BaL were harvested, centrifuged at 400 x g to remove cells and stored in aliquots of 500 µl at -80°C.

2.4 Determining titre of virus stocks

The titres of infectious viral stocks, expressed as the 50% tissue culture infectious dose (TCID₅₀), were determined following the culture of serial end-point dilutions of viral stocks of NL4-3 and BaL onto C8166 and C8166-R5 cells, respectively. After thawing, C8166 and C8166-R5 cells were resuscitated and propagated in complete RPMI 1640. After approximately 1 week of log phase growth, the cell density was calculated by counting in a Kova chamber using Trypan Blue to determine the number of live cells/ml, centrifuged at 400 x g for 5 minutes to form a cell pellet and re-suspended at a density of 3.33×10^5 cells/ml in fresh complete RPMI 1640. A 96-well flat-bottomed tissue culture plate was marked so that the central 48 wells (8 horizontal and six vertical) were used and 150µl of the cell suspension was seeded into replicate wells of the flat-bottomed plate. Serial 10-fold dilutions of each viral stock were prepared in complete RPMI 1640 from 10⁰

(undiluted virus) to a dilution of 10^{-6} ; 50 μ l of each dilution was then added to each of the 6 replicate wells from 10^0 to 10^{-6} plus a negative cell control, such that each well contained 200 μ L. Sterile PBS was added to the empty wells around the perimeter of the plate to reduce “edge effects” resulting from evaporation. Plates were incubated at 37°C in 5% CO₂. After 4 days, cells were examined for cytopathic effects (CPE) by counting the number of syncytia in cells in each well (>3 syncytia per well assessed as positive). The TCID₅₀ was calculated using the Reed-Muench formula (i.e. to determine the viral dilution when 50% of well become infected) for both NL4-3 and BaL viruses and from which the multiplicity of infection (MoI) was calculated;

MoI = Plaque forming units (pfu) of virus used for infection / number of cells

pfu/ml = $0.7 \times \text{TCID}_{50}$

2.5 SYBR Green Performance Enhanced Reverse Transcription (SG PERT) Assay

2.5.1 Viral lysis

The SG-PERT assay was used to determine the relationship between RT activity and viral titre. Serial 10-fold dilutions of viral stocks of NL4-3 and BaL were prepared in complete RPMI 1640, as previously described and 5 μ l of the viral dilutions were mixed with 95 μ l of lysis buffer and incubated for 10 minutes.

2.5.2 Reverse transcription (first round of SG PERT assay)

A reverse transcription (RT) master mix was prepared for n+1 reactions. The volumes shown below are for each reaction.

5XFS reaction buffer	4 μ l
dNTP mix	1 μ l
Primer MS2-Reverse 10 μ M	1 μ l
0.1M DTT	2 μ l
RNAse Out	1 μ l
MS2 RNA (1 in 40 dilution)	1 μ l
Double distilled water	5 μ l

15 µl of RT master mix were dispensed into 0.2 ml PCR tubes. After incubation for 10 minutes with lysis buffer (see section 2.1.5.1), 5 µl of the viral lysate was added to each RT reaction and mixed by pipetting up and down 4 times. The PCR tubes were capped and reactions were transferred to the thermal cycler and incubated as follows:

Reverse transcription	37°C for 30 minutes
Inactivation	95°C for 5 minutes
Hold	4°C indefinitely

2.5.3 Real-time PCR (second round of SG-PERT assay)

A real-time PCR master mix was prepared for n+1 reactions. The volumes shown below are for each reaction.

SYBR green reaction buffer	10 µl
Primer MS-Forward 10µM	0.6 µl
Primer MS-Reverse 10µM	0.6 µl
Double distilled water	3.8 µl

15 µl of the real-time PCR master mix was dispensed into 0.2 ml white well PCR tubes and 5 µl of each of the first round PCR products were added to the wells containing real-time PCR master mix. Bio-rad optical caps were applied to the white well strips and samples amplified on a Bio-Rad real-time thermal cycler under the following conditions:

Hot start	95°C for 15 minutes	1 cycle
Denature	94°C for 15 seconds	} 45 cycles
Anneal	60°C for 30seconds	
Extend	72°C for 30 seconds	
Hold	4°C	indefinitely

Amplification curves were generated with Bio-Rad software allowing the crossing point (C_q) (i.e the cycle number at which the fluorescence of a sample rises above the background fluorescence) to be calculated for a given dilution of virus stock and from

which standard curves can be generated. For the standard curve, a linear relationship between the dilutions of the viral stocks and RT activity as measured on the SG PERT assay are demonstrated in **Figure 4.2**. An approximation of the dilution required to achieve a viral titre equivalent to a MoI of 1.5 could then be determined.

2.6 Determining the viral inhibitory concentrations of C34-PEG₄-Chol

The IC₅₀ and a 90% inhibitory concentration (IC₉₀) of C34-PEG₄-Chol was determined in a panel of viruses including NL4-3 by the Pharmaceutical company and found to be 0.05 nM; independent verification of this result was sought prior to further investigations.

TZM-bl cells, in log phase growth, were prepared by incubation with trypsin for approximately 5-8 minutes, centrifugation and then re-suspending in complete DMEM medium. Cells were counted and diluted to a density of approximately 10⁵ cells/ml in complete DMEM. After resuspension, 100 µl of TZM-bl cells were added to wells of two 96-well plate (equivalent to 10⁴ cells/well) and incubated overnight. The following morning, serial 10-fold dilutions of NL4-3 viral stock were prepared from 10⁰ (undiluted) to 10⁻³ in complete DMEM medium. Supernatant from TZM-bl cells was removed and 10 µl of each viral dilution was added in triplicate to the wells and made up to 100 µl by adding 90 µl of complete DMEM (therefore equivalent to 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ dilutions of virus stock); diluted virus were allowed to adsorb onto the TZM-bl cells for 3-4 hours.

In the interim, dilutions of C34-PEG₄-Chol were prepared between 0.05 nM and 25.6 nM. After 3-4 hours of incubation with TZM-bl cells, virus supernatant was removed from each well and cells were washed with 200 µl of sterile PBS. Wells were then loaded with 200 µl of each dilution of drug in DMEM. Infected cells were then incubated for 72-hours at 37°C, 5% CO₂ (**Figure 2.1**).

Figure 2.1: Plate set up for determining IC₅₀ and IC₉₀ of C34-PEG₄-Chol

Plate 1

	0nM	0nM	0nM	0.05nM	0.05nM	0.05nM	0.1nM	0.1nM	0.1nM	0.2nM	0.2nM	0.2nM
10 ⁰												
10 ⁻¹												
10 ⁻²												
10 ⁻³												
	0.4nM	0.4nM	0.4nM	0.8nM	0.8nM	0.8nM	1.6nM	1.6nM	1.6nM	3.2nM	3.2nM	3.2nM
10 ⁰												
10 ⁻¹												
10 ⁻²												
10 ⁻³												

Plate

	6.4nM	6.4nM	6.4nM	12.8nM	12.8nM	12.8nM	25.6nM	25.6nM	25.6nM			
10 ⁰												
10 ⁻¹												
10 ⁻²												
10 ⁻³												

After incubation for 72- hours, the supernatant containing drug was removed from each well and replaced with 100 μ l Promega lysis buffer (diluted 1/5 with nuclease-free water (MiliQ)). Plates were then frozen at -80°C for 2 hours. After this time, plates were removed from the CL3 laboratory and thawed to determine relative infectivity of each well with cells in the presence of drug. Luciferase assay buffer was thawed and reconstituted by adding 10 mls buffer to one vial of substrate. 50 μ l of lysate was then transferred to white 96-well plates and mixed with 50 μ l of substrate. Plates were then read on the luminometer adjusting gain to 4000. Percentage (%) inhibition of luminescence in the presence of each concentration of drug was then calculated as a surrogate marker of relative infection of each well of cells in the presence of drug; % inhibition was plotted against drug concentration results and IC₅₀ and IC₉₀ of C34-PEG₄-Chol inferred for NL4-3.

2.7 Selection of C34-PEG₄-Chol resistant HIV variants

PM-1 cells were counted using a Kova chamber, centrifuged at 500 x g for 5 minutes and resuspended at a density of 5 x 10⁵ cells/ml. Following resuspension, 0.5 ml cells were seeded in 4 replicate wells on a 24 well plate (a control and an intervention well for both viral clones). Serial 10-fold dilutions of NL4-3 and BaL stocks were undertaken to between 10⁻⁴ and 10⁻⁵ (approximately equivalent to Cq 30-32) with complete RPMI 1640 and 1 ml of viral dilutions were allowed to adsorb onto the cells to achieve a MoI of approximately 1.5 and incubated at 37.0°C, 5% CO₂. To select C34-PEG₄-Chol resistant variants, after approximately 4 hours, 1 ml culture medium was removed and replaced with complete RPMI 1640 containing C34-PEG₄-Chol and incubated at 37.0°C, 5% CO₂ for 48-72 hours. Viral replication was assessed by monitoring for CPE; in addition, RT activity was measured by intermittently collecting viral supernatant and performing SG PERT assays, as described previously. Following communication with the peptide manufacturer, owing to slowing of hydrolysis of the peptide at an acidic pH induced by cellular metabolism, it was estimated after 72 hours of culture, between 50-80% of peptide would remain intact. Therefore after 72 hours of culture, cells were expanded with complete RPMI 1640 containing fresh C34-PEG₄-Chol to maintain drug pressure within the culture medium and incubated for a further 48-72 hours at 37.0°C, 5% CO₂. Viral supernatants were then harvested and centrifuged at 400 x g for 5 minutes to remove cellular debris and aliquoted for freezing at -80.0°C. The RT activity of the supernatant was measured by SG PERT, as previously described, to estimate the viral titre; based upon the Cq on SG PERT, serial 2-fold dilutions then undertaken to dilute the virus to approximately a Cq of 30-32

(approximating a TCID₅₀ of the original stock virus). The diluted viral supernatants were then used for subsequent rounds of infection using the same protocol but with a higher concentration (usually 1.5-2 fold) of C34-PEG₄-Chol.

2.8 RNA extraction, reverse transcription and amplification by nested PCR

2.8.1 Extraction of RNA from culture supernatants

Only viral culture supernatants harvested on the final day of exposure to C34-PEG₄-Chol at a given concentration were sequenced. RNA from NL4-3 supernatants exposed from 0.05-12.8 nM and BaL exposed from 0.05-25.6 nM of C34-PEG₄-Chol were extracted.

RNA was extracted from the culture supernatants according to the protocol as described in the QIAamp® Viral RNA Mini Handbook (Third edition, April 2010). For each culture sample to be sequenced, 560 µl of the kit lysis buffer (AVL containing carrier RNA) was mixed with 140 µl of cell-free supernatant in a microcentrifuge tube and pulse-vortexed for 15 seconds and incubated at room temperature for 10 minutes, rendering the virus non-infectious. After incubation, 560 µl of ethanol (96–100%) was added to each sample, and mixed by pulse-vortexing for 15 seconds. 630 µl of the solution was then added to the QIAamp Mini column (in a 2 ml collection tube) and centrifuged at 6000 x g for 1 minute. The filtrate was discarded and the QIAamp Mini column was placed into a new 2 ml collection tube; this was then repeated until all the supernatant was filtered through the QIAamp Mini column. 500 µl of AW1 buffer was added to each QIAamp Mini column, centrifuged at 6000 x g for 1 minute and the filtrate discarded. The QIAamp Mini columns were then placed into clean 2 ml collection tubes and 500 µl of AW2 buffer added and centrifuged at 20,000 x g for 3 min. Finally, the QIAamp Mini columns were placed in clean 1.5 ml microcentrifuge tubes and add 60 µl of AVE buffer added to each column, incubated at room temperature for 1 minute and centrifuge at 6000 x g for 1 minute. Purified filtrates were then frozen at -80°C until sequencing was performed.

2.8.2 Oligonucleotide primer design

gp41 specific primers were designed with reference to the Los Alamos HIV databases and compendiums (HXB2 Consensus of Consensus sequence, found at <http://www.hiv.lanl.gov/content/sequence/HIV/CONSENSUS/Consensus.html>) (Table 2.1) for use in nested PCR. Primers were ordered through Sigma-Aldrich on a

synthesis scale of 0.05 μ mole, purified by HPLC and delivered as lyophilised pellets in tubes for reconstitution with double distilled nuclease free water to a stock concentration of 100 μ M and then diluted to 10 μ M for use within PCRs.

Table 2.2: Forward and reverse oligonucleotide primers for reverse transcription, amplification and sequencing of gp41

Name	Residue co-ordinate	Direction	Primer sequence
gp41-1	7350-7372	Forward	TTTAATGTGGAGGGGAATTTT
gp41-2	9070-9089	Reverse	TCCAGTCCCCCTTTCTT
gp41-3	7651-7670	Forward	GGGACAATGGAGAAGTGAA
gp41-4	9063-9082	Reverse	CCCCCTTTCTTTTAAAAAG
gp41-5	7992-8014	Forward	CAACAGCTCCTGGGGATTTGGGG
gp41-6	8417-8438	Reverse	TCCACCTTCTTCTTCTATTCCT

2.8.3 Reverse transcription-PCR

A reverse transcription master mix was prepared for n+1 reactions in the “pre-PCR” room

2X master mix	25 µl
gp41 primer-1	1 µl
gp41 primer-2	1 µl
RT enzyme mix	1 µl
Double distilled water	12 µl

For each RNA extraction, 39 µl of RT-PCR master mix was dispensed into 0.2 ml PCR vials and 10 µl of purified sample added and mixed by pipetting up and down four to five times. For reverse amplification reactions, samples were then transferred to the “post-PCR” room and cycled in the thermal cycler under the following conditions:

55°C, 30 minutes	1 cycle
94°C, 2 minutes	1 cycle
94°C, 30 seconds	} 30 cycles
55°C, 30 seconds	
68°C, 1 minute	
68°C, 5 minutes	1 cycle
4°C , indefinitely	1 cycle

2.8.4 Nested PCR

For the second round of the nested PCR, a master mix was prepared for n+1 reactions (volumes shown are per reaction):

Nucleotide mix (10mM)	1 µl
Reaction (Gold) buffer	5 µl
MgCl ₂ (25mM)	3 µl
gp41 primer-3	0.5 µl
gp41 primer-4	0.5 µl
Taq Gold polymerase	0.25 µl
Double distilled water	38.75 µl

For each reaction, 48 µl of the master mix was dispensed into 0.2 ml PCR tubes to which 1 µl of the first round products were added using a multi-channel pipette and mixed 4-5 times. The sequences were then amplified in the thermal cycler under the following conditions:

94°C, 8 minutes	1 cycle
94°C, 30 seconds	} 30 cycles
55°C, 30 seconds	
72°C, 1 minute	
72°C, 8 minutes	1 cycle
4°C, indefinitely	hold

The second round PCR products were stored in a refrigerator at 4°C until confirmation of product amplification on agarose gel.

2.8.5 Agarose gel electrophoresis

Amplification of products was confirmed by visualization following agarose gel electrophoresis. Agarose gels were prepared by adding 1 g of agarose to 100 ml of Tris base/Boric acid/EDTA (TBE) buffer into a conical flask and boiling in a microwave for 1 minute until the agarose was completely dissolved. The agarose gel was then allowed to cool to around 65°C before adding 10 µl of SYBR-safe solution and poured into a mould with a 12-tooth comb to create wells within the gel and allowed to set. Once set, the gel was transferred to an electrophoresis tank containing TBE buffer to a depth of about 0.5 cm above the gel and the comb removed. Samples were then applied by mixing 5 µl of each PCR product with 1 µl of loading buffer in a microtitre plate and adding 5 µl of each sample to individual wells. In addition, 1 µl of molecular weight markers were mixed with 10 µl of TBE buffer and 2 µl of loading buffer and 5 µl of the mixture added to the final well. The gel with PCR products and molecular weight markers was run for 1 hour at 120 volts. Gels were subsequently visualised and digitally photographed on an ultraviolet plate within an illumination box. Upon confirmation of amplification of gp41 within samples, the second round products from the PCR were sent for full length Sanger sequencing of gp41 using gp41 primers 3-6 inclusive, as follows (courtesy of Dr Steve Kaye and Mr Simon Dustan, Molecular Diagnostic Unit, Jefferiss Trust Laboratories, Imperial College London).

2.8.6 Purification of PCR products

Ultracel columns were assembled and 400 μ l double-distilled water were pipetted into the upper chamber. Second round PCR products were pipetted into the upper chamber, the column was capped and centrifuged at 3000g for 10 minutes. A further 40 μ l of double distilled water was added into the upper chamber, the column removed from the collection tube, inverted and placed in clean collection tube. Products were centrifuged for 2 minutes at 500 g and used immediately.

2.8.7 Sequencing reactions

Sequencing reactions were carried out using Applied Biosystems BigDye Terminator v3.1 sequencing kits. A master mix of terminator ready reaction mix, primer and water was prepared in a 1.5ml microcentrifuge tube as follows (volumes shown are per reaction):

Terminator ready reaction mix	4.0 μ l
Sequencing primer	0.5 μ l
Molecular grade water	1.5 μ l
Purified PCR product	6.0 μ l

The master mix was inverted multiple times to mix and 6 μ l aliquoted into 0.2ml PCR reaction tubes. Subsequently 4 μ l of template was added to each reaction tube to make a total volume of 10 μ l. PCR reaction vials were then thermal cycled (in post-PCR area) under the following conditions:

96°C, 1 minute	1 cycle
96°C, 10 seconds	} 25 cycles
50°C, 5 seconds	
60°C, 4 minutes	
4°C , indefinitely	1 cycle

Prior to removal of samples from the thermocycler, 2 solutions were prepared as follows:

1. A sodium acetate/ethanol solution with 1µl sodium acetate 3M and 25µl absolute ethanol per reaction.
2. A 70% ethanol solution made with absolute ethanol and deionised water containing enough for 75µl per reaction.

26µl of sodium acetate/ethanol solution was added to each reaction, the PCR tubes were sealed and reactions mixed by multiple inversions. Reaction tubes were centrifuged at 2000 x g for 20 minutes. The caps of the PCR tubes were removed and lint-free tissue placed on top and the plate inverted and centrifuged at 150 x g for 5 minutes. After 5 minutes, the tissue was removed and 75µl of 70% ethanol added to each tube and reactions centrifuged at 2000 x g for a further 5 minutes. The PCR caps were removed again and lint-free tissue placed on top and the plate inverted followed by centrifuging at 150 x g for 1-2 minutes.

2.8.8 Sequencer set up

Applied Biosystems 3100-Avant Capillary Electrophoresis Genetic Analyser was used for sequencing along with data collection software. Forty millilitres of running buffer was prepared by a 1 in 10 dilution of 10X buffer with molecular grade water. The capillary was removed from the sequencer and placed upright with the ends immersed in buffer. Polymer blocks were removed from the connecting tube, allowing upper and lower blocks to be separated. Reservoirs were rinsed with molecular grade water and fresh solutions placed according to the diagram below (**Figure 2.2**):

Figure 2.2:

BACK

Pos 2 – Water (rinse)	Pos 4 – Water (waste)
Pos 1 – Buffer (cathode reservoir)	Pos 3 – Water (spare)

FRONT

The two polymer blocks were rinsed with molecular grade water driven through the tubes by a syringe, blocks were dried by pushing air through the tubes using a new, dry syringe and drying completed with white, lint-free tissues. Syringes were rinsed by drawing up and expelling molecular grade water several times. A small amount of polymer was drawn into each syringe and drawn back to coat the barrel with polymer and subsequently expelled. The required amount of polymer was then drawn into each syringe and replaced back onto the sequencer. The sequencer doors were opened, rinsed and the anode reservoir dried and filled with fresh buffer and subsequently calibrated according to a standard operating procedure.

2.8.9 Sample application

Samples were re-suspended with 10µl Hi-Di Formamide (HDF); 10µl were added to each sample, tubes were centrifuged and allowed to stand for 10-15 minutes. While the samples were standing, plate records were set to heat to 96 °C; samples were applied to the thermal cycler plate at 96°C, heated for 2 minutes and removed when cooled to 4°C for approximately 10 minutes. The sample plate was then placed onto the 3100 analyser and started.

2.8.10 Assembly and editing of sequence data

At the end of the sequencer run the data (chromatograms) were stored and “Sequencher version 5.4.1” software was later used to edit sequence chromatograms. To edit:

- From “File” open “New project from template”, choose “in-house resistance” option.
- From “File” click “import sequences” and browse to the run data folder. Import the four sequences needed for the first sample. Highlight the four sequences and select “Trim ends” from the “Sequence” menu. Trim the ends.
- Highlight the four sequences together with the reference sequence (gp41) and click the “assemble to reference” button.
- Highlight the Contig [0001] icon and from the drop-down “Contig” menu click “Trim to reference sequence”. From the “Sequence” menu open “Call secondary peaks”, set to 20%.
- Double click the Contig [0001] icon, and click on “Bases” and “Show chromatograms” in the new window.

- Sequences were edited by scrutinizing sequence mismatches (indicated by “+”), ambiguities (indicated in bold blue or pink font) and gaps (indicated by “:”).
- The amino acid sequence was scrutinised to ensure no stop codons were present.

When the editing was complete, the consensus sequence was exported to the completed projects folder: FASTA files were generated and analysed using Bioedit software version 7.2.5.

2.9 Construction of double-stranded DNA template for site-directed mutagenesis

2.9.1 Restriction digestion of pcDNA6/V5-His B and pNL4-3

The plasmids, pcDNA6/V5-HisB (Thermofisher) and pNL4-3, were used to generate the template pcDNA6/V5-HisB_gp41 for site-directed mutagenesis. Both plasmids contain the common endonuclease restriction enzyme sites, Nhe1 and BamH1 (New England Biolabs). Restriction digestion reactions were set up to extract the gp41 insert from pNL4-3 and linearise the pcDNA6/V5-His B vector:

<u>Insert</u>		<u>Vector</u>	
pNL4-3	10 µl	pcDNA6/V5-His B	10 µl
Cutsmart buffer	5 µl	Cutsmart buffer	5 µl
BamH1	3 µl	BamH1	3 µl
Nhe1	3 µl	Nhe1	3 µl
Double-distilled water	29 µl	Double distilled water	29 µl

Reactions were set up in 1.5 ml microcentrifuge tubes, then incubated at 37 °C for 3 hours on a heat-block.

2.9.2 Gel purification of digestion fragments

A 1% agarose gel was prepared by adding 1 g of agarose to 100 ml of Tris base/acetic acid/EDTA (TAE) buffer and heated on full power in a microwave in a conical flask for 1 minute, allowed to cool to around 65 °C and poured into a mould and allowed to set with 3 large tooth comb to create wells in the gel. Once set, the gel was transferred into an electrophoresis tank with TAE buffer, the comb was removed and the digestion products were mixed with 10 µl of loading buffer before applying the products to the gel (with molecular weight markers in the third well). The gel was run for 1 hour at 120 volts,

after which the digestion products were visualised on an ultraviolet plate and molecular weight markers used to identify the linearised pcDNA6/V5-His B and gp41 fragments. Fragments were excised from the gel using a scalpel and placed into 1.5 ml microcentrifuge tubes for gel of DNA fragments.

2.9.3 Extraction of DNA digestion products from gel bands

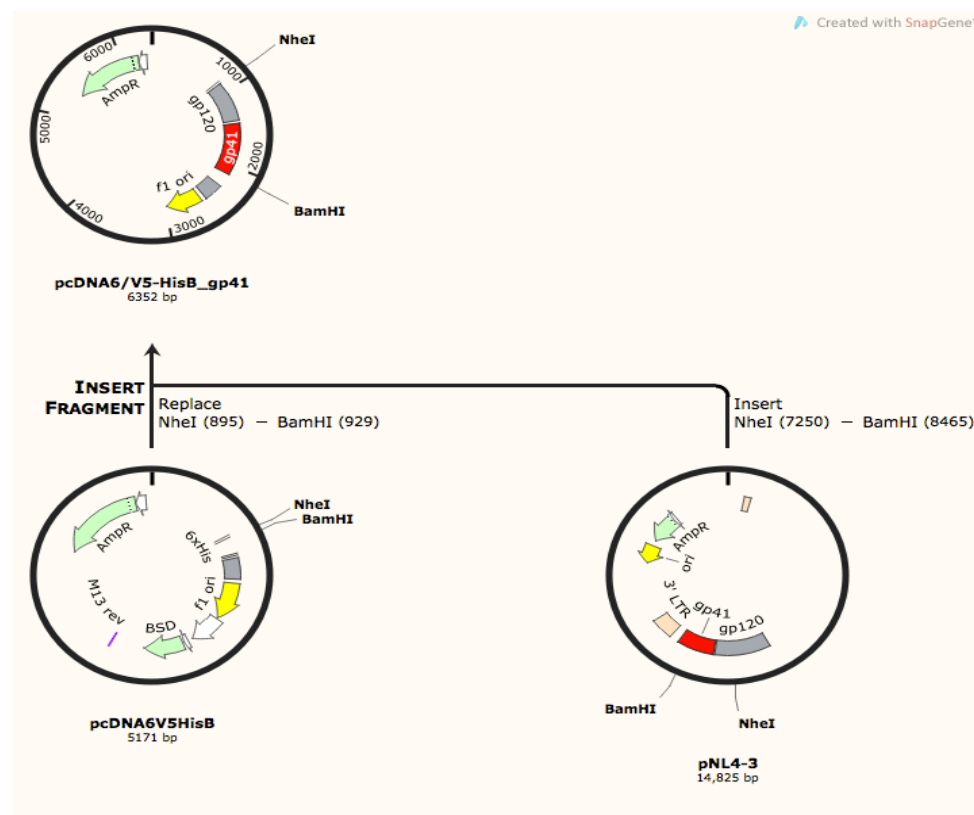
Extractions were performed using an Illustra GFX Gel Band Purification Kit ®. The weight of the gel bands was calculated and 10 µl of the capture buffer type were added for each 10 µg of agarose gel slice and incubated on a hot block at 60 °C until the agarose bands had dissolved. The capture buffer/sample mix was then applied to an assembled microspin column, incubated for 1 minute and then centrifuged at 16 000 x g for 30 seconds; the procedure was repeated until all the capture buffer/sample mix was loaded and the filtrate was subsequently discarded (to collect the DNA fragments in the membrane of the microspin column). The sample was washed by adding 500 µl of wash buffer to the microspin column, centrifuging at 16 000 x g for 30 seconds and dried by placing the column into a new collection tube and centrifuging at 16 000 x g for a further 30 seconds. The DNA fragments were then captured by addition of 30 µl of an elution buffer and repeating the centrifugation at 16 000 x g and retaining the eluent. The concentrations (in ng/ml) of the gp41 and linearised pcDNA6/V5-His B fragments was then determined by photospectrophotometry.

2.9.4 Ligation of linearised pcDNA6/V5 His B and gp41

Following gel purification the digestion products were ligated using T4 DNA ligase to ligate the insert gp41 into the vector linearised pcDNA6/V5-His B; the insert and vector were then ligated in a ratio of 1 ng: 2 ng ratio in a 10 µl reaction and incubated overnight at room temperature in a 1.5 ml microcentrifuge tube (**Figure 2.3**):

10 X DNA ligation buffer	1 µl
T4 DNA ligase	1 µl
Linearised pcDNA6/V5 His B	3 µl
gp41 fragment	5 µl
Total	10 µl

Figure 2.3: Synthesis of pcDNA6/V5-His B_gp41 template plasmid



A fragment of the *env* gene (corresponding to co-ordinates 7250-8465) from the HIV-1 plasmid, pNL4-3, including sequences from the gp120 V3 through the gp41 intracellular domain was digested using the endonuclease restriction enzymes *NheI* and *BamHI* and ligated into pcDNA6/V5-HisB to create pcDNA6/V5-His B_gp41.

2.9.5 Transformation of pcDNA6/V5-His B_gp41 into competent cells and expansion

Following ligation, a stock of methylated pcDNA6/V5-His B_gp41 was generated by transformation of the ligation products into TOP 10 competent cells, a Dam⁺ E. coli strain (Invitrogen). The TOP 10 cells were removed from storage at -80°C and gently thawed in their vial on ice. After thawing, 1 µl of the ligation product was added to the vial of TOP 10 cells and incubated on ice for 30 minutes. After incubation, the TOP 10 cell/ligation product mixture was heat-shocked at 42°C for 45 seconds, then placed on ice for a further 2 minutes before adding 100 µl of SOC medium pre-warmed to 42°C. The cells were placed in a shaker for 1 hour and then spread onto an ampicillin containing agar plate and incubated at 37°C, 5% CO₂ overnight. Thereafter, 4 bacterial colonies were grown by adding the individual colonies to 4 separate 14 ml BD propylene tubes containing 4 ml each of Luria Bertani broth (LB) (with 0.1% ampicillin). Cultures were placed in a shaker at 37 °C overnight.

2.9.6 Extraction of pcDNA6/V5-His B_gp 41 from bacterial cultures

The following morning, plasmids were extracted from bacterial cultures using Thermo-Fisher GeneJet™ Plasmid Miniprep kit. The cultures were first transferred to 15 ml conical Falcon tubes and centrifuged at 4000 x g for 15 minutes to form bacterial pellets. The spent LB was discarded and the pelleted bacteria re-suspended in 250 µl of re-suspension solution (containing RNase A). The bacterial suspension was then transferred to a microcentrifuge tube and 250 µl of lysis solution were added and mixed by inverting the tube several times until the solution becomes viscous and more clear. Neutralization solution (350 µl) were added and centrifuged for 5 minutes to pellet cell debris and chromosomal DNA. The supernatant was then added to the spin column by decanting and centrifuged for 1 minute and the flow-through was discarded. Wash solution (500 µl) were added to the column and centrifuged for 60 seconds and the flow-through was discarded. The wash procedure was repeated and the flow-through discarded, followed by centrifugation at 16 000 x g for an additional 1 minute to remove residual wash solution from the spin column membrane (in order to avoid residual ethanol in plasmid preps). The spin column was then transferred into a fresh 1.5 ml microcentrifuge tube and 50 µl of the elution buffer was added to the spin column, incubated for 2 minutes and then centrifuged for 2 minutes at 16 000 x g to collect the prepared pcDNA6/V5 His B_gp41 plasmids. Concentration of the pcDNA6/V5 His B_gp41 plasmid was determined by

spectrophotometry using the elution buffer to blank the spectrophotometer prior to measurement.

2.9.7 Confirmation of gp41 insertion into pcDNA6/V5_His B_gp41 construct

Following the plasmid miniprep, construction of the pcDNA6/V5-His B_gp41 template was confirmed by restriction digestion of the template and agarose gel electrophoresis of the template (pcDNA6/V5-HisB acting as a control):

<u>Template digestion</u>		<u>Control digestion</u>	
pcDNA6/V5-HisB_gp41	3 µl	pcDNA6/V5-His B	3 µl
Cutsmart buffer	2 µl	Cutsmart buffer	2 µl
Nhe1	0.2 µl	Nhe1	0.2µl
BamH1	0.2 µl	BamH1	0.2 µl
Double-distilled water	14.6 µl	Double-distilled water	14.6 µl

Digestion reactions were incubated at 37°C in a 1.5 ml microcentrifuge tube for 3 hours and subsequently applied to 1% agarose gel and run for 1 hour, as previously described in section 2.9.1. Direct visualisation under ultraviolet light confirmed the separated bands (gp41 and pcDNA6/V5-His B) on the agarose gel.

2.10 Site-directed mutagenesis

2.10.1 Mutagenic primer design

The design of the mutagenic primers was based on the reference sequence, NL4-3. Mutations introduced within the primer design were chosen from those observed following *in vitro* passage of BaL with C34-PEG₄-Chol and also from those most commonly observed in clinical practice following treatment failure with enfuvirtide. Two complimentary mutagenic oligonucleotide primers were designed for each mutation, such that the desired mutation was present within both primers and to allow the same sequence to anneal on opposite ends of the plasmid. Mutations were located centrally within the primer and flanked on either side by unmodified nucleotide sequence of between 18-22 nucleotides (so that total length of the mutagenic primer was between 40-44 nucleotides

long). Primers were designed to contain a GC content of around 40% with the aim of a melting temperature of $\geq 78^{\circ}\text{C}$. The primers were obtained from Sigma-Aldrich on a synthesis scale of 0.05 μmole , purified by HPLC and delivered as lyophilised pellets in tubes for reconstitution with double distilled nuclease-free water to a stock concentration of 100 μM , then diluted to 10 μM for use in PCRs.

2.10.2 Site-directed mutagenesis

Mutant strand synthesis reactions were undertaken according to Stratagene Site-Directed Mutagenesis reaction protocols.

Sample and control reactions were prepared in 0.2 ml PCR tubes as follows:

<u>Sample reaction</u>		<u>Control reaction</u>	
pcDNA6/V5-HisB_gp41	x μl	pcDNA6/V5-HisB_gp41	x μl
10 $\times$ reaction buffer	5 μl	10 $\times$ reaction buffer	5 μl
Oligonucleotide primer 1	125 μl	Double distilled water	250 μl
Oligonucleotide primer 2	125 μl	dNTP mix	1 μl
dNTP mix	1 μl		

Both reactions were then made up to a final volume of 50 μl with double distilled water before addition of 1 μl of PfuUltra HF DNA polymerase (2.5 U/ μl) and thermocycled. Cycle reactions were conducted according to the following cycling parameters:

95°C, 30 seconds	1 cycle
95°C, 30 seconds	} 16 cycles
55°C, 1 minute	
68°C, 1 minute/kb plasmid length	
4°C, indefinitely	hold

Following thermocycling, reactions were placed on ice for 2 minutes.

2.10.3 *DpnI* digestion of amplification products

Following thermocycling, *DpnI* was added to sample and control products to digest the parental pcDNA6/V5-His B_gp41 plasmids and leave behind the mutated pcDNA6/V5-His B_gp41 plasmids. Exactly 1 μ l of *DpnI* was added to each reaction and mixed thoroughly by pipetting up and down multiple times, spun in a microcentrifuge for 1 minute and incubated on a heat block for 1 hour at 37°C (**Figure 2.4**).

Figure 2.4: Schematic of sequence within site-directed mutagenesis reaction

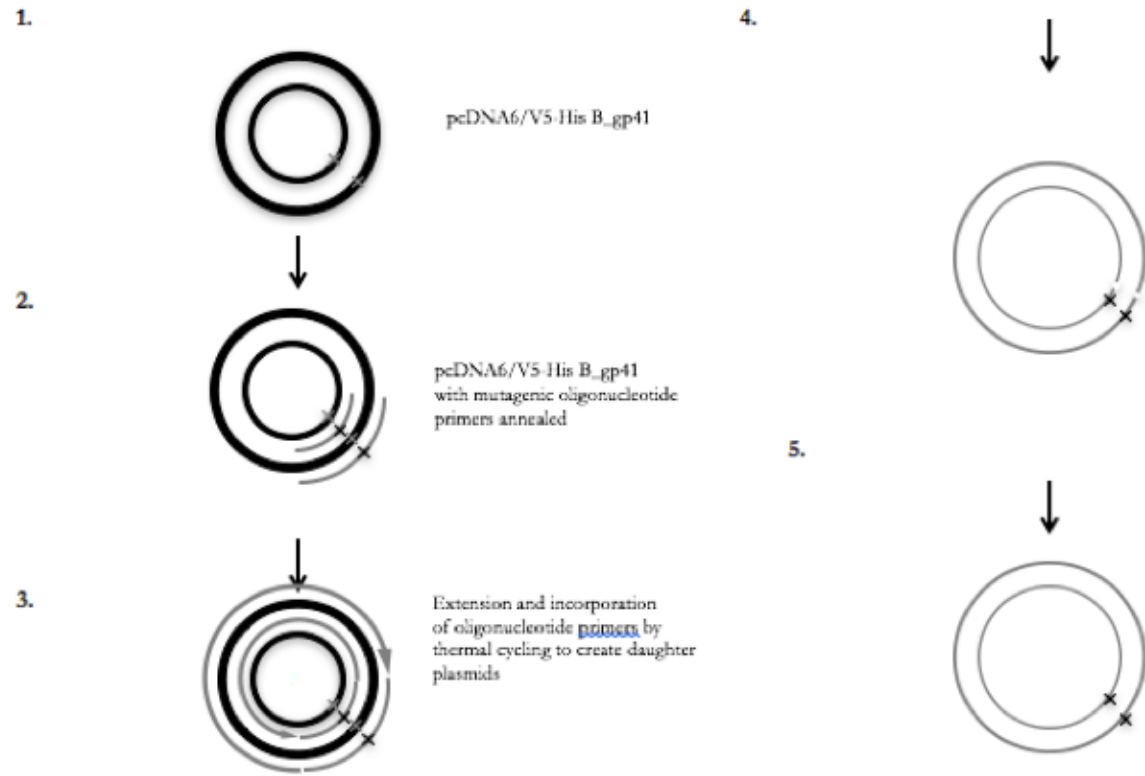


Fig 2.4 1. Methylated plasmid preparation –pcDNA6/V5-His B_gp41 – gp41 incorporated into pcDNA6/V5-His B plasmid containing target site for mutation. **2.** Thermal cycling – pcDNA6/V5-His B is denatured and mutagenic oligonucleotide primers containing the desired mutations anneal. **3.** Thermal cycling – DNA polymerase extends and incorporates the mutagenic primers, resulting in nicked strands in a daughter unmethylated mutated plasmid. **4.** *Dpn* I used to digest out the non-mutated methylated parental pcDNA6/V5-His B_gp41 plasmid. **5.** Unmethylated mutated daughter pcDNA6/V5-His B_gp41 is transformed into competent cells which repair the nicked ends in the mutated plasmid. *Adapted from: QuikChange site-directed mutagenesis kit instruction manual (Revision A, Copyright 2006).*

2.10.4 Transformation of XL1-Blue Supercompetent Cells

XL1-Blue supercompetent cells were subsequently transformed with *Dpn1* digestion products mutated plasmids according to Stratagene Site-Directed mutagenesis protocols. Cells were removed from storage at -80°C and gently thawed on ice in a polystyrene box. For each mutagenic reaction, 50 µl of cells were then aliquoted into two prechilled 14 ml BD Falcon polypropylene round-bottomed tubes; 1 µl of *Dpn1* digestion products from the sample and control reactions were then aliquoted into each of the polypropylene tubes containing XL1-Blue supercompetent cells, gently swirled and then incubated on ice for 30 minutes. Reactions were then heat-shocked by immersion of polypropylene tubes in a water bath at 42°C for 45 seconds and put back on ice for a further 2 minutes. Subsequently, 500 µl of SOC medium preheated to 37°C was added to each tube and incubated in a shaker at 37 °C for 1 hour at 225-250 rpm. After approximately 1 hour, 250 µl of the transformation reactions were plated onto separate ampicillin agar plates and incubated overnight at 37 °C. The following morning, plates were removed from the incubator and inspected for the presence (sample reactions) and absence (control reactions) of bacterial colonies. The expected colony number for successful reactions was between 10 and 200 colonies per plate whereas control reactions should have no colonies present. Plates were then placed in a cold room until inoculation of LB for expanding the cultures.

2.10.5 Expansion of bacterial colonies and extraction of mutated pcDNA6/V5-HisB_gp41

Four 14 ml BD Falcon polypropylene round-bottomed tubes were prepared with 4 ml of LB supplemented with 4 µl of ampicillin (100 µl/ml). Four bacterial colonies (without surrounding satellite colonies) were selected from the sample ampicillin agar plate; colonies were harvested using a sterile plastic loop and each colony was inoculated separately into the prepared propylene tubes containing LB. Tubes were then placed in a shaker overnight at 37 °C at 225-250 rpm. The following morning, bacterial cultures were grown to extract the mutated pcDNA6/V5-HisB_gp41 plasmids as described in section 2.1.9.6 and concentration of the plasmid confirmed using spectrophotometry. Mutated plasmids were subsequently sent for sequencing to confirm the presence of the desired mutation(s), as described in section 2.1.8.7.

2.11 Generation of NL4-3 with point mutations

2.11.1 Sub-cloning of gp41 into pNL4-3Δgp41

2.11.1.1 Restriction digestion to extract mutated gp41 from pcDNA6/V5-His B_gp41 and linearise pNL4-3

After sequencing to confirm that the desired mutation had been integrated into gp41, the mutated pcDNA6/V5_His B_gp41 plasmid was digested using *Nhe*I and *Bam*HI to extract gp41. Simultaneously, pNL4-3 underwent restriction digestion to obtain pNL4-3Δgp41 for ligation with the mutated gp41:

pcDNA6/V5-HisB_gp41	3 µl	pNL4-3	3 µl
Cutsmart buffer	2 µl	Cutsmart buffer	2 µl
<i>Nhe</i> I	0.2 µl	<i>Nhe</i> I	0.2 µl
<i>Bam</i> HI	0.2 µl	<i>Bam</i> HI	0.2 µl
Double-distilled water	14.6 µl	Double-distilled water	14.6 µl

Digestion reactions were incubated at 37°C in a 1.5 ml microcentrifuge tube for 3 hours and subsequently applied to 1% agarose gel and run for 1 hour, as previously described in section 2.1.9.2. Direct visualisation under ultraviolet light was used to confirm separation of the bands (gp41 and pcDNA6/V5-His B) on the agarose gel and segments were purified from the agarose gel using an Illustra GFX Gel Band Purification Kit, as described in section 2.1.9.3.

2.11.1.2 Ligation of mutated gp41 with linearised pNL4-3Δgp41

Following gel purification, the digestion products were ligated using T4 DNA ligase to ligate the mutated gp41 into pNL4-3Δgp41; the insert and vector were ligated in a ratio of 1 ng: 2 ng ratio in a 10 µl reaction and incubated overnight at room temperature in a 1.5 ml microcentrifuge tube:

10 X DNA ligation buffer	1 µl
T4 DNA ligase	1 µl
Linearised pNL4-3Δgp41	X µl
Mutated gp41 fragment	X µl
Total	10 µl

2.11.1.3 Transformation of mutated pNL4-3 into competent cells

A stock of mutated pNL4-3 was generated by transformation of the ligation products into TOP 10 competent cells. As previously described, TOP 10 cells were removed from storage at -80°C and gently thawed in their vial on ice. After thawing, 1 µl of the ligation product was added to the vial of TOP 10 cells and incubated on ice for 30 minutes. After incubation, the TOP 10 cell/ligation product mixture was heat shocked at 42°C for 45 seconds, then placed on ice for a further 2 minutes before adding 100 µl of pre-warmed SOC medium to 42°C. The cells were placed in a shaker for 1 hour and then spread onto an ampicillin agar plate and incubated at 37°C overnight. Following overnight incubation, 4 bacterial colonies were expanded by adding the individual colonies to 4 separate 14 ml BD propylene tubes containing 4 ml each of LB (with 0.1% ampicillin). Cultures were placed in a shaker at 37 °C overnight.

2.11.1.4 Extraction of mutated pNL4-3 from recombinant bacterial cultures

After overnight incubation, bacterial cultures were grown to extract the mutated pNL4-3 plasmids, as described in section 2.1.9.6 and the concentration of the plasmid confirmed by spectrophotometry.

2.11.1.5 Small scale digest to confirm mutated gp41 insert present within mutated pNL4-3 plasmid

Following miniprep, a small scale digest was undertaken to confirm ligation of the gp41 into pNL4-3Δgp41. A mastermix was prepared in a 1.5 ml microcentrifuge tube to a volume of 17µl of plasmid and mixed with 3 µl of plasmid, as follows (volumes given for each reaction):

Cutsmart buffer	2 µl per
Nhe1	0.2 µl
BamH1	0.2 µl
Double-distilled water	14.6 µl
Mutated pNL4-3	3 µl

Reactions were then incubated on a heat-block at 37 °C for 3 hours. After 3 hours, the digestion products were applied to an agarose gel prepared as described previously. The agarose gel was prepared by adding 1 g of agarose to 100 ml of Tris base/acetic acid/EDTA (TAE) buffer in a conical flask and boiling in a microwave for 1 minute until the agarose was completely dissolved. The agarose gel was then allowed to cool to around 65°C before adding 10 µl of SYBR-safe solution and poured into a mould with a 12-tooth comb to create wells within the gel and to allowed to set. Once set, the gel was transferred to an electrophoresis tank containing TAE buffer to a depth of about 0.5 cm above the gel and the comb was removed. Samples were then applied to the gel by mixing 5 µl of the small scale digestion products with 1 µl of loading buffer in a microtitre plate and adding 5 µl of each sample to individual wells; in addition, 1 µl of markers were mixed with 10 µl of TAE buffer and 2 µl of loading buffer and 5 µl of the mixture added to the final well. The gel with PCR products and molecular weight markers was then run for 1 hour at 120 volts. Gels were then visualised and digitally photographed on an ultraviolet plate within an illumination box for separation of gp41 from pNL4-3.

2.11.1.6 Sequencing of positive clones

Plasmid preparations identified as having integrated gp41 were sequenced using primers BGH-reverse and TP7-forward, as described previously.

2.11.2 Transfection of mutated pNL4-3 plasmid to generate viral stock

Plasmids were transferred into CL3 laboratories for transfections to generate mutated NL4-3, as previously described. Briefly, one day prior to transfection, 3×10^5 HEK 293T cells were plated onto a large cell culture plate in the presence of complete DMEM; cells were allowed to grow to 70-90% confluence. On the day of transfection, 300 µl incomplete DMEM “serum free medium” were mixed with 6 µg of mutated pNL4-3 in 15ml polystyrene Falcon tube followed by the addition of 18 µl of PEI 1 µg/1 ml.

Solutions were pulse-vortexed 15 times for 1 second each and incubated for 20 minutes at room temperature. The old medium was then removed from the HEK 293T cells and cells washed with PBS followed by dripping 700 μ l of pNL4-3/PEI/DMEM solution to cover the entire well. After incubation of the cell/transfection mixture for a further 3 hours, 2 ml of complete DMEM was added and incubated for a further 2 days at 37°C, 5% CO₂. Approximately 48 hours later, culture supernatants was collected from the wells using a 5 ml pastette; supernatants were centrifuged at 500 x g for 5 minutes and separated into 500 μ l aliquots before freezing at - 80°C.

2.11.3 Expansion of mutated NL4-3

Mutated NL4-3 was expanded as previously described. Briefly, PM-1 cells were thawed, resuscitated and propagated in complete RPMI 1640. After 1 week of growth, cells were centrifuged to form a cell pellet and re-suspended at a density of 5×10^5 cells/ml in complete RPMI 1640. One ml of viral supernatant was then allowed to adsorb onto 500 μ l of the re-suspended PM-1 cells (total 2.5×10^5 cells) for between 1 and 4 hours at 37 °C, 5% CO₂ in a 6 well plate. After incubation for 1-4 hours, 1 ml of culture medium was removed and replaced with fresh culture medium and incubated for a 72 hours at 37 °C, 5% CO₂. After this time, the infected cells were counted and half the infected PM-1 cells were transferred to a T-25 cell culture flask; approximately a 4-fold excess of uninfected PM-1 cells were then added to the infected cells and suspended in 5-6 ml of complete RPMI 1640 cell culture medium overnight and kept upright in a T-25 flask. The following morning, the culture was made up to 18 ml with complete RPMI 1640 and incubated for a further 4 days. After this time, the viral supernatants containing mutated NL4-3 were harvested, centrifuged at 400 x g to remove cells and stored in aliquots of 500 μ l at -80°C. The titre of the recombinant mutated NL4-3, expressed as TCID₅₀, was determined following serial endpoint dilutions of the recombinant viral stock and onto C8166, as described in section 2.1.4.

2.12 Determining phenotypic resistance inferred by a point mutation in gp41

TZM-bl cells, in log phase growth, were prepared by adding trypsin for approximately 5-8 minutes, centrifugation and then re-suspension in complete DMEM medium. Cells were counted and diluted to a density of approximately 10^5 cells/ml in complete DMEM. After resuspension, 100 μ l of TZM-bl cells were added to wells of two 96-well plate (equivalent

to 10^4 cells/well) and incubated overnight. The following morning, serial 10-fold dilutions of the mutated NL4-3 viral stock were made from 10^0 (undiluted) to 10^{-3} in complete DMEM medium. Supernatant from TZM-bl cells was removed and 10 μ l of each viral dilution were added in triplicate to wells and made up to 100 μ l by adding 90 μ l of complete DMEM (therefore equivalent to 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} dilutions of viral stock); viral dilutions were allowed to adsorb onto the TZM-bl cells for 3-4 hours.

In the interim, dilutions of C34-PEG₄-Chol were prepared between 0.05 nM and 25.6 nM. After 3-4 hours of incubation with TZM-bl cells, viral supernatant was removed from each well and cells were washed with 200 μ l of sterile PBS. Two hundred μ l of each dilution of drug in DMEM were then applied to each well. Infected cells were incubated for 72-hours at 37°C, 5% CO₂ (**Figure 2.1**).

After incubation for 72-hours, the supernatant-containing drug was removed from each well and replaced with 100 μ l Promega lysis buffer (diluted 1/5 with nuclease-free water (MiliQ)). Plates were then frozen at -80°C for 2 hours, then removed from the CL3 laboratory and thawed to determine relative infectivity of each well with cells in the presence of drug. Luciferase assay buffer was thawed and reconstituted by adding 10 mls buffer to one vial of substrate. Lysate (50 μ l) was transferred to white 96-well plates and mixed with 50 μ l of substrate. Plates were read on the luminometer adjusting gain to 4000. Percentage (%) inhibition of luminescence in the presence of each concentration of drug was calculated as a surrogate marker of relative infection of each well of cells in the presence of drug; % inhibition was plotted against drug concentration results and IC₅₀ and IC₉₀ of C34-PEG₄-Chol inferred for mutated NL4-3.

2.13 Resistance testing of *ex vivo* samples

For participants enrolled in the ‘first-in-man study’, plasma was collected pre-dosing and on days 14 and 28 post-dosing for gp41 sequence analysis to assess for the presence of resistance associated mutations. Samples were collected from participants in 6ml EDTA blood tubes. Within 1 hour of collection, samples were centrifuged at 2000 x g for 10 minutes and separated into aliquots of 1 ml in a microbiological safety cabinet at the Jefferiss Trust Laboratories and stored at -80°C until the time of sequencing. Unblinding of clinicians was planned at the end of the trial such that only samples from participants who received C34-PEG₄-Chol would be analysed and performed in a batch.

RNA was extracted from plasma using QIAamp Viral RNA Mini Kit as per standard protocols. Briefly, 1.0ml plasma was pipetted into a labelled 'V' bottom Sarstedt vial. If less than 1.0ml plasma was available, the volume was made up to 1.0ml with PBS and mixed gently by pipetting. Vials were centrifuged for 1 hour at 25,000 x g, 4°C. Supernatant (860µl) was removed using a 1ml pipette. Subsequently, 560µl Qiagen lysis buffer AL was added to each vial and vortexed. The Qiagen extraction procedure was continued according to the manufacturer's instructions, making the final elution in 60µl buffer AVE. The RNA extract was kept on ice until ready to add to the RT-PCR. RT, amplification and sequencing was then performed discussed in section 2.1.8.

Chapter 3

Clinical methods

3.1 Study set up

A study protocol was developed by collaboration between the Clinical Trials team and Statistical Trials team based at Imperial Clinical Trials Unit (ICTU). Details of the trial design were published at: <http://www.isrctn.com/ISRCTN89747147>. The trial was jointly funded by the Medical Research Council (MRC) and JV Bio and was sponsored by Imperial College London. Clinical Trial Authorisation was obtained following protocol submission to the Medicine and Healthcare Regulatory Authority (MHRA) and ethical approval for conduct of the study was obtained from the London – Central Research Ethics Committee. Finally, local approval for the study was obtained from Imperial College Joint Research Compliance Office (JRCO).

3.2 Participants

Participants were recruited from two hospital Trusts (Imperial College Healthcare Trust, St. Mary's and Chelsea & Westminster NHS Foundation Trust, West London Centre for Sexual Health). Enrolment into the study had to occur within 28 days of a screening visit. Eligible participants were men aged 18-60, living with HIV for ≥ 6 months and not receiving ART. Due to publication of results from the START study advocating universal ART for all PLWHIV, only those who opted to defer starting ART for the duration of the clinical trial and who had discussed ART deferral with their primary HIV physician were considered eligible. In addition, only those with an absolute CD4 count ≥ 400 cells/ μL and HIV RNA $\geq 10,000$ copies/mL and who were assessed as being unlikely to require ART within the following 4 months were eligible. Participants had to have no clinically significant findings following examination and for reproducibility of pharmacokinetic studies, a body mass index (BMI) between 19-28 inclusive was mandated. Any co-morbidities, if present, had to be optimally managed. Heterosexual participants had to be willing to use two effective forms of contraception from screening until the end of the study. Haematology and chemistry results had to be within predefined limits and patients had to have no clinically significant abnormalities on electrocardiogram (ECG).

Exclusion criteria included any active or serious medical or psychiatric illness. Those with current alcohol use considered likely to interfere with the conduct of the study or those who had used any recreational drugs within 14 days of screening were excluded; urine was also screened for recreational drugs on the day of dosing and if positive, those participants were not randomised. Smoking was permitted, providing this remained constant

throughout the study. Participants were not allowed to have taken part in another clinical trial of an investigational medicinal product (CTIMP) within 90 days of screening. The only concomitant medications permitted included paracetamol and multivitamins; those who had received ARV drugs within the preceding 6 months or those who had ever received enfuvirtide in the past were ineligible. Any clinically significant resistance-associated mutations within *pol* also rendered participants ineligible.

3.3 Study design

The study was a phase 1 (two stage) first-in-man, double-blind, randomised placebo-control, single centre trial of monotherapy (single dose or multi-dose) with C34-PEG₄-Chol. Only development of stage 1 of the study is described within this thesis.

Although all proposed substages of the trial are described, only substage 1 (including dose groups 1 and 2) were implemented owing to discontinuation of the trial due to adverse events.

3.4 Objectives

The aim of stage 1 was to assess the safety, tolerability and pharmacokinetic profile of C34-PEG₄-Chol following a single subcutaneous dose in men living with HIV who were not currently receiving ART. To this end within stage 1, three substages were planned:

- Substage 1 – a dose escalation and safety phase to assess the safety of the proposed dose range of 10 – 80 mg.
- Substage 2 – an adaptive phase to determine the optimal dose and dose interval (not implemented).
- Substage 3 – optional cohort to improve estimation of the dose-response curve for the optimal dose and dose interval from substage 2 (not implemented).

From results within stage 1, the optimal dose and dosing interval to bring forward into the multi-dose stage 2 of the study would be determined.

3.5 Outcome measures

Primary and secondary outcome measures for stage 1 were defined within the study protocol prior to initiation of the clinical trial.

3.5.1 Primary outcome measures

Primary outcomes were defined by safety and pharmacokinetic parameters.

Safety parameters included:

- Grade 3 or above eosinophilia.
- Grade 3 or above injection site reactions.
- Grade 3 or above clinical or laboratory adverse events.
- Any serious adverse event which could be attributable to C34-PEG₄-Chol.

The pharmacokinetic parameter was:

- The lowest dose of C34-PEG₄-Chol which achieves a plasma concentration 10 times above the IC₅₀ at the furthest time point after administration of C34-PEG₄-Chol.

3.5.2 Secondary outcome measures

Secondary outcome measures for the clinical trial included:

- Any adverse event (grade 1 and above).
- Detailed pharmacokinetic profile of C34-PEG₄-Chol following single administration at different doses.
- Changes in plasma HIV RNA.

3.6 Sample size

A maximum of 42 participants were planned for enrollment across the 2 study stages (twenty-eight participants in stage 1, fourteen participants in stage 2). Across both stages, a sample size comprising 31 receiving active drug and 11 receiving placebo (n=42) was considered sufficient to give 80% power to observe adverse events which have a population incidence of $\geq 30\%$. Of 28 participants to be enrolled into stage 1, 20 were to receive active drug and 8 were to receive placebo.

3.7 Structure of stage 1

Allocation to active drug or placebo was through a process of simple randomisation and

was only planned for substage 1; within substages 2 and 3, all participants would receive active drug.

3.7.1 Substage 1

Within substage 1, randomisation of patients within four sequential dose groups (n=4 per dose group; therefore a total of sixteen participants) was undertaken via computer generated lists. Randomisation was double-blind to active drug or placebo:

- Dose group 1 10 mg (2 participants C34-PEG₄-Chol, 2 participants placebo)
- Dose group 2 20 mg (2 participants C34-PEG₄-Chol, 2 participants placebo)
- Dose group 3 40 mg (2 participants C34-PEG₄-Chol, 2 participants placebo)
- Dose group 4 80 mg (2 participants C34-PEG₄-Chol, 2 participants placebo)

Randomisation was undertaken using Inform Integrated Trial management system and was on a 1:1 basis with no predefined stratification. A decision to dose escalate was to be made only after all 4 participants in each dose cohort had been observed for a minimum of 7 days. All safety data were reviewed by the independent data monitoring committee (IDMC).

3.7.2 Substage 2

The Bayesian adaptive substage of the study (n=10) was planned to commence on assumption that the 10-80 mg dose range was found to be safe after substage 1. Within substage 2, it was planned that ten participants would receive active drug at any of the eight doses under consideration (10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg). Dose allocation was based on a continual reassessment method (CRM). Dosing was planned to be conducted in five dose groups where 2 patients at a time would receive the same dose of active drug. Decision on dose adjustments were planned based on the clinical and statistical rules described in chapter 6.

3.7.3 Substage 3

In order to improve modelling of the dose-response curve, a final cohort of 2 patients (n=2) was proposed to receive the dose considered optimal based on pharmacokinetic data obtained across substages 1 and 2.

3.8 Study procedures

3.8.1 Interventions

The active peptide C34-PEG₄-Chol was formulated in accordance with good manufacturing practice (GMP) as described in section 2.2 by the American Peptide Company whilst packaging and release of the compound was through Symbiosis Pharmaceutical Services. The product was prepared as a white lyophilised powder, stored at -20°C +/- 5°C and reconstituted with 5mL of sterile water for injection. Sodium chloride 0.9% acted as an isotonic placebo. The choice of starting dose was determined as described in section 1.3.3.3.3.

3.8.2 Screening visit

The screening visit occurred between 7 and 28 days before study entry. This visit included a detailed review of study procedures and potential risks of taking part in the study before signing an informed consent form. In addition, a detailed medical history, including history of previous ARV drug exposure was collected and patients underwent a complete physical examination and assessment of potential injection sites. Blood samples were collected for haematology, biochemistry, plasma HIV RNA, T-cell count and, where not previously available, a baseline HIV RNA resistance profile including for gp41 and *pol*. An ECG was performed as part of the baseline investigations to exclude any underlying cardiac arrhythmia and for comparison during follow up in the clinical trial to assess for cardiotoxicity of C34-PEG₄-Chol (**Table 3.1**).

3.8.3 Dosing visit

Within 7-28 days of screening, eligible participants were admitted to an inpatient clinical research unit (Imperial Clinical Research Facility at Hammersmith Hospital) at the beginning of the treatment period for assessments of safety parameters and pharmacokinetic assessments for 24 hours post-dose delivery. At multiple time points throughout day 0, participants had a check of vital signs, a symptom-directed physical examination and review of injection sites. Sampling for haematology (full blood picture) and chemistry (electrolytes, creatinine, estimated glomerular filtration rate and liver enzymes). Plasma HIV RNA was sampled pre-dosing. Additionally, an ECG was performed at 4 hours, 12 hours and 24 hours post-dosing.

Intensive pharmacokinetic, haematology and chemistry blood sampling was undertaken pre-dose and post-dosing at 30 mins, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours and 24 hours. For ease of sampling, all samples were collected via venous cannulae inserted at the time of admission and sampling was synchronised with the time of administration of drug.

3.8.4 Follow up visits

The remaining visits for all dose groups were conducted on an outpatient basis at 48 hours and days 3, 4, 7, 10, 14, 21, 28 and 84. At all follow-up visits, participants had a further review of adverse events, any concomitant medications, a check of vital signs, a symptom-directed physical examination, review of injection sites and sampling for pharmacokinetic analysis and haematology and chemistry. HIV RNA was sampled on days 4, 7, 14, 21 and 28 post-dosing. Samples were taken for sequence analysis of gp41 to monitor emergence of resistance to the drug and these were collected on days 14 and 28.

Table 3.1: Clinical study schedule of visits and assessments

Day	-28 to -7 days	Day 0	Day1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14	Day 21 (+/- 3 days)	Day28 (+/- 3 days)	Day 84 (+/- 3 days)
		inpatient										
Phase of study	Screening	Dosing		Post dosing safety, follow up and pharmacokinetic analysis								
Medical history	X											
Concomitant/recreational drugs	X		X	X	X	X	X	X	X	X	X	X
Physical examination ^a	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Adverse events		X	X	X	X	X	X	X	X	X	X	X
Eligibility criteria	X	X										
Injection site review ^b	X			X	X	X	X	X	X	X	X	X
Haematology/chemistry ^c	X			X	X	X	X	X	X	X	X	X
Lymphocyte subsets	X											
Inpatient PK ^d												
Outpatient PK				X	X	X	X	X	X	X	X	X
Plasma HIV RNA	X	X	X			X	X		X		X	X
HIV resistance profile	X								X		X	

^a Full physical examination at screening and symptom directed examination thereafter.

^b Assessment of injection site reactions pre-dose and at 1 h, 4 h and 12 h post dosing.

^c Full blood picture, creatinine and electrolytes, liver function tests, glucose and amylase taken pre-dose and at 2 h, 4 h, 8 h, 12 h post dose on day 0 and day 1.

^d Intensive (inpatient) PK sampling pre-dose and at 30 mins, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h on day 0 and 24 h post dose on day 1.

3.8.5 Safety intervals

To assess for stopping rules, a minimum interval of 4 days was planned between each cohort of 2 patients within each dose group; the choice of 4 days was pragmatically based on allometric scaling from rhesus monkeys and indicated that the likely plasma half-life would be around 72 hours. In addition, prior to dose escalation between dose groups, a review was planned by the Trial Steering Committee (TSC) to assess safety.

3.8.6 Reconstitution of the drug

Depending on dose allocation, participants were to receive either one or two subcutaneous injections; the volume of placebo was matched with that of the active drug and both were indistinguishable in appearance. Active drug or placebo was reconstituted and administered by two unblinded nurses on the morning of dosing according to the volumes in **Table 3.2**.

Table 3.2: Reconstitution of IMP with water for injections: volumes per dose

Dose	Volume of injection(s)		
10 mg	0.5 mL		
20 mg	1.0 mL		
30 mg	1.5 mL		
40 mg	2.0 mL		
50 mg	1.5 mL	+	1.0 mL
60 mg	1.5 mL	+	1.5 mL
70 mg	2.0 mL	+	1.5 mL
80 mg	2.0 mL	+	2.0 mL

3.9 Pharmacokinetic analysis

Blood samples from each time-point were centrifuged at 2000g x 10 minutes within 1 hour of collection; plasma samples were separated from whole blood and stored at -80°C. Samples were sent in batches on dry ice to the University of Liverpool at pre-specified time points and analysed by high-performance liquid chromatography (HPLC) on a Thermo Access mass spectrometer in positive ion sim mode and an Accela HPLC system equipped with Xbridge BEH 3100 C18 column eluting with 0.1% TFA in acetonitrile. The

lower limit of quantification for the assay was 62.5 ng/mL and the inter and intra assay variability did not exceed 10%. Pharmacokinetic parameters were derived via non-compartmental analysis of plasma C34-PEG₄-Chol from day 0 to day 84. The area under the curve was calculated following a single dose, assuming linear decrease over time, via linear trapezoidal methods. The C_{max} was the maximum concentration observed at each dose and the T_{max} was the time taken to reach the maximum plasma concentration after dosing.

3.10 Toxicity assessments

Clinical and laboratory adverse events associated with previous studies on the use of enfuvirtide in TORO-1 and -2 studies were reviewed and graded against the Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Events, version 2.0 (November 2014), available at:

http://rsc.techres.com/Document/safetyandpharmacovigilance/DAIDS_AE_Grading_Table_v2_NOV2014.pdf

For grading of injection site reactions (ISRs), a grading system based on the DAIDS table and from reports within the TORO-1 and -2 studies was devised (**Table 3.3**); community representatives were consulted to gain consensus on a partially devised grading system based on acceptability of toxicities in a contemporary situation.

Table 3.3: Grading of injection site reactions

	Mild (Grade 1)	Moderate(Grade 2)	Severe (Grade 3)	Potentially Life Threatening (Grade 4)	Rationale for grading	Frequency of mild/moderate ISRs with T-20 in pivotal Phase 3 studies†
INJECTION SITE REACTIONS						
Pain	Does not interfere with activity	Repeated use of non-narcotic pain reliever > 24 hours or interferes with activity	Any use of narcotic pain reliever or prevents daily activity	Emergency department visit or hospitalization	Preventive Vaccine Clinical Trials grading scale	93.1
Tenderness	Mild discomfort to touch	Discomfort with movement	Significant discomfort at rest	Emergency department visit or hospitalization	Preventive Vaccine Clinical Trials grading scale	89.8
Erythema / Redness *	0.5-2.5 cm lasting >24 hours after dosing	2.6 - 10 cm at any time post dosing during follow up	> 10 cm at any time post dosing during follow up	Necrosis or exfoliative dermatitis at any time post dosing during follow up	Adapted from Preventive Vaccine Clinical Trials grading scale	90.8
Induration / Swelling **	0.5-2.5 cm lasting >24 hours after dosing AND does not interfere with activity	2.6 - 10 cm at any time post dosing during follow up OR interferes with activity	> 10 cm at any time post dosing during follow up OR prevents daily activity	Necrosis at any time post dosing during follow up	Adapted from Preventive Vaccine Clinical Trials grading scale	94.4
Nodules/cysts	0.5-1.0 cm	1.1-1.5 cm	1.6-2.0 cm	NA	In the absence of internationally agreed grading system trial specific grading system has been devised	81.6
Pruritus associated with injection	Itching localized to injection site AND Relieved spontaneously or with < 48 hours treatment	Itching beyond the injection site but not generalized OR Itching localized to injection site requiring ≥ 48 hours treatment	Generalized itching causing inability to perform usual social & functional activities	NA	DAIDS grading table	Not reported
Bruising	0.5-1.0 cm	1.1-1.5 cm	1.6-2.0 cm	>2.0 cm or haematoma	In the absence of internationally agreed grading system trial specific grading system has been devised	Not reported

* In addition to grading the measured local reaction at the greatest single diameter, the measurement should be recorded as a continuous variable.

** Induration/Swelling should be evaluated and graded using the functional scale as well as the actual measurement.

† Percentages are based on T-20 vs. Optimised Regimen Only Studies (TORO-1 and TORO-2), Phase III studies comparing the efficacy and safety of 24 weeks of treatment with enfuvirtide in combination with an optimised background antiretroviral regimen (OBR + T-20) with the efficacy and safety of optimised background therapy alone (OBR alone).

3.11 Stopping criteria

As participants received only one dose of the drug during stage 1, no stopping criteria were applied for individual participants; however, in order to prevent further participants being exposed to doses that are potentially toxic, stopping criteria for specific doses within stage 1 were agreed prior to the start of the clinical trial:

- In the event of ≥ 1 serious adverse events (SAEs) at a specific dose, which could be attributable (possibly, probably or definitely) to C34-PEG₄-Chol, administration of further doses was to be put on hold until reviewed by the trial management group. If deemed that the SAE was definitely, probably or possibly related to C34-PEG₄-Chol, no further doses were to be administered to any participants at this dose level or above.
- If ≥ 2 participants experienced a severe intensity event (grade 3 or 4 clinical or laboratory event confirmed on attendance or repeat testing) at a specific dose, which could be attributable (possibly, probably or definitely) to C34-PEG₄-Chol, further doses at this level or above were to be halted.
- If ≥ 3 or more participants experience a moderate intensity event (grade 2 clinical or laboratory event confirmed on attendance or repeat testing) at a specific dose, which could be attributable (possibly, probably or definitely) to C34-PEG₄-Chol, further doses at this level or above were to be halted.

Chapter 4

**Evolution of mutations in HIV-1 gp41
during viral culture with C34-PEG₄-Chol**

4.1 Introduction

Genetic variability in the HIV quasi species can affect the ability of a drug regimen to maintain viral suppression and the presence of such drug-resistant virus is an independent predictor of treatment failure. Consequently, understanding the genetic pathways to ARV resistance is critical in constructing an effective drug regimen for a patient. The efficacy of an ARV regimen depends on the susceptibility of the viral quasi species to individual components of a regimen and usually involves targeting at least two stages of the HIV lifecycle. As a molecular definition, HIV mutations refer to changes in the nucleic acid sequence compared to a reference sequence, such as NL4-3 or HXB2 and result from a rapid replication rate and lack of proof reading ability of RT. Variability in genetic sequences can also be generated by genetic recombination when viruses of different sequences enter a susceptible cell. Such changes in the nucleic acid sequence may be silent, or result in a change in the primary amino acid sequence and, in turn, the secondary protein structure; thereafter, the functionality of the protein may be altered. Furthermore, immune escape acts as a driver in the introduction of further genetic variability. Neutralisation epitopes reside on the surface of both gp120 and transmembrane gp41 envelope glycoproteins. HIV can be neutralized when antibodies bind to critical domains within the envelope glycoproteins thus disrupting the process of viral entry. However, through introduction of genetic variability in the envelope, epitopes which may be vulnerable to neutralization by antibodies and T-cell responses avoid recognition of host immune responses thus allowing an infection to persist. Multiple rounds of neutralization escape are thought to explain, in part, how infection persists, a mechanism which has been recognized in several other lentiviruses [144]. Polymorphisms refer to naturally occurring mutations which occur in this way, not selected by drugs but which can influence drug susceptibility. Conversely, “primary” mutations appear in response to drug exposure and are first in the resistance pathway acting through a direct effect on drug binding and present at or near an active site. Primary mutations may be either acquired or transmitted, but equally are the inevitable consequence of incomplete suppression of viral replication by a drug. As a result of changes in the structure or function of proteins, a fitness cost to the virus may be induced rendering the virus less replication competent. To overcome this effect of primary mutations, “secondary” mutations may ensue, which although they do not usually confer drug resistance in their own right, may compensate for the initial fitness defects and modulate susceptibility. While changes in the conformation of structural proteins may result in a lower replication rate of the virus, mutations in the nucleotide

sequence allow a viral quasi species to emerge which can replicate more competently in the presence of that drug than the wild type virus [145]. With removal of this drug pressure, however, wild type virus will again predominate within the quasi species, with resistant variants persisting and archived at low levels in latently infected cells.

For some ARV drugs, multiple drug-resistant mutations are required to induce resistance whilst for others a single point mutation may induce high-level resistance. The number of amino acid substitutions required for the development of resistance to each ARV drug has been termed the “genetic barrier to resistance”. Moreover, certain amino acid substitutions inducing resistance may emerge more readily if a single nucleotide substitution within an amino acid codon results in a different amino acid being substituted into a primary protein sequence. For example, the Methionine-184-Valine (M184V) mutation requires only a single nucleotide substitution within the codon (from alanine to guanine) to result in a substitution of amino acid and render a virion highly resistant to lamivudine and emtricitabine:

Methionine	Valine
AUU	GUU
AUC	GUC
AUA	GUA
AUG	GUG

Identification of mutations is usually determined through *in vitro* selection, phase I studies and site-directed mutagenesis. Most assays for assessing ARV resistance are based on sequencing of viral genes (genotyping) to identify those mutations known to confer reduced drug susceptibility. For HR-2 based FIs, most notably enfuvirtide, mutations conferring resistance have most commonly occurred within the HR-1 domain of gp41. As part of the development of C34-PEG₄-Chol, gaining an understanding of mutations conferring reduced susceptibility to the drug is critical to its potential future clinical use. Given the overlap of the peptide C34 with enfuvirtide, it is speculated that mutations within the HR-1 domain will also occur within the HR-1 domain.

Hypothesis

In the presence of residual HIV viral replication, drug pressure will result in the selection of mutations in the HR-1 region of gp41.

Aims

To this end, the aims of this chapter were:

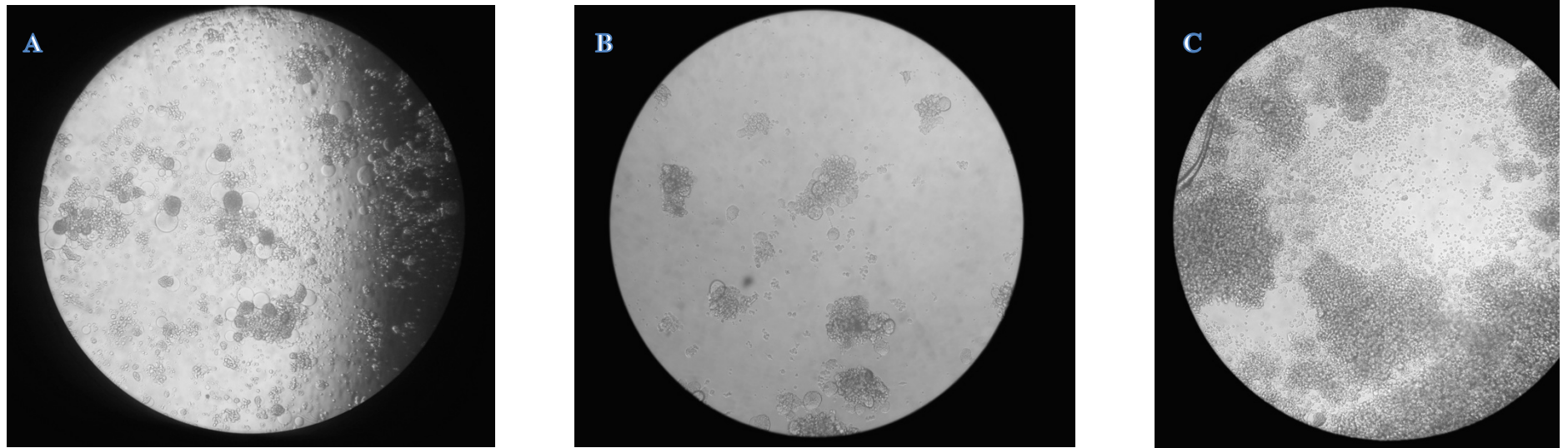
1. To determine the IC_{50} and IC_{90} of C34-PEG₄-Chol.
2. To culture HIV in the presence of incrementing concentrations of C34-PEG₄-Chol to induce evolution of a viral species resistant to C34-PEG₄-Chol.
3. To sequence the full length of gp41 using oligonucleotide primers to identify mutations which may be responsible for drug resistance.

4.2 Results

4.2.1 Calculation of the viral titre

Viral stocks of NL4-3 and BaL were generated as described in section 2.1.3. Both viruses were then cultured in the cell line PM-1, which is susceptible to infection by both X4- (NL4-3) and R5- (BaL) tropic viruses. Following centrifugation to separate viral supernatants, the virus titre was calculated as the 50% tissue culture infectious dose (TCID₅₀); the TCID₅₀ is the tissue culture infectious dose which will infect 50% of the cell monolayers challenged with the defined inoculum. This was determined by performing serial 10-fold dilutions of each stock and undertaking replicate cultures of the serial dilutions in C8166 and C8166-R5 cells in flat-bottomed 96-well plates. After 72 hours had elapsed, replicate cultures were examined. Infectivity end-points were determined microscopically according to the emergence of syncytia in both C8166 and C8166-R5 cells; flat bottomed wells were considered positive if three or more syncytia appeared in each well after 72 hours. The TCID₅₀ was calculated by the Reed-Muench formula according to the number of positive wells in each replicate culture (**Figure 3.1**). As expected, syncytia and the number of positive wells became less common with higher dilutions of virus. The TCID₅₀/mL was calculated as 2.9×10^5 TCID₅₀/mL for NL4-3 (**Table 3.1**) and 6.04×10^5 TCID₅₀/mL for BaL (**Table 3.2**).

Figure 4.1 Syncytia formation in C8166 cells with serial dilutions of virus following 72 hours of culture



C8166 T-lymphoblastoid cells growing in culture viewed at x100 magnification; characteristically cells grow in large clumps. The above figures represent the same cell batch acutely infected with NL4-3 with consecutive 10-fold viral dilutions and incubated for 72 hours; large multinucleated cells are produced when sufficient viral protein is expressed on the surface of cells allowing fusion of cell membranes. A = cells incubated for 72 hours with NL4-3 diluted to 10^{-3} , B= cells incubated for 72 hours with NL4-3 diluted to 10^{-4} , C= cells incubated for 72 hours with NL4-3 diluted to 10^{-5} .

Table 4.1 **NL4-3 viral stock titre via Reed-Muench calculation**

	Observed values		Accumulated values		
Virus dilution	Wells +	Wells -	Wells +	Wells -	% infected
10⁻³	6	0	10	0	100
10⁻⁴	3	3	4	3	57.1
10⁻⁵	1	5	1	8	12.5
10⁻⁶	0	6	0	14	0
10⁻⁷	0	6	0	20	0

50% endpoint lies between 10⁻⁴ and 10⁻⁵ dilutions:

$$\begin{aligned}
 & \frac{[(\% \text{ infected wells at next dilution above } 50\%) - (50\%)]}{[(\% \text{ infected wells at next dilution above } 50\%) - (\% \text{ infected wells at next dilution below } 50\%)]} \\
 & = \frac{[57.1 - 50]}{[57.1 - 12.5]} \\
 & = 0.16 \\
 \text{TCID}_{50}/50\mu\text{L} & = 10^{4.16}/50\mu\text{L} \\
 \text{TCID}_{50}/\text{mL} & = 10^{4.16} \times 20 \\
 \text{TCID}_{50}/\text{mL of NL4-3 stock} & = 2.9 \times 10^5 \text{ TCID}_{50}/\text{mL}
 \end{aligned}$$

Table 4.2 BaL viral stock titre via Reed-Muench calculation

	Observed values		Accumulated values		
Virus dilution	Wells +	Wells -	Wells +	Wells -	% infected
10^{-3}	6	0	12	0	100
10^{-4}	5	1	6	1	83.3
10^{-5}	1	5	1	6	14.2
10^{-6}	0	6	0	12	0
10^{-7}	0	6	0	18	0

50% endpoint lies between 10^{-4} and 10^{-5} dilutions:

$$\begin{aligned}
 & \frac{[(\% \text{ infected wells at next dilution above } 50\%) - (50\%)]}{[(\% \text{ infected wells at next dilution above } 50\%) - (\% \text{ infected wells at next dilution below } 50\%)]} \\
 & = \frac{[83.2 - 50]}{[83.3 - 14.2]} \\
 & = 0.48 \\
 \text{TCID}_{50}/50\mu\text{L} & = 10^{4.48}/50\mu\text{L} \\
 \text{TCID}_{50}/\text{mL} & = 10^{4.48} \times 20 \\
 \text{TCID}_{50}/\text{mL of BaL stock} & = 6.04 \times 10^5 \text{ TCID}_{50}/\text{mL}
 \end{aligned}$$

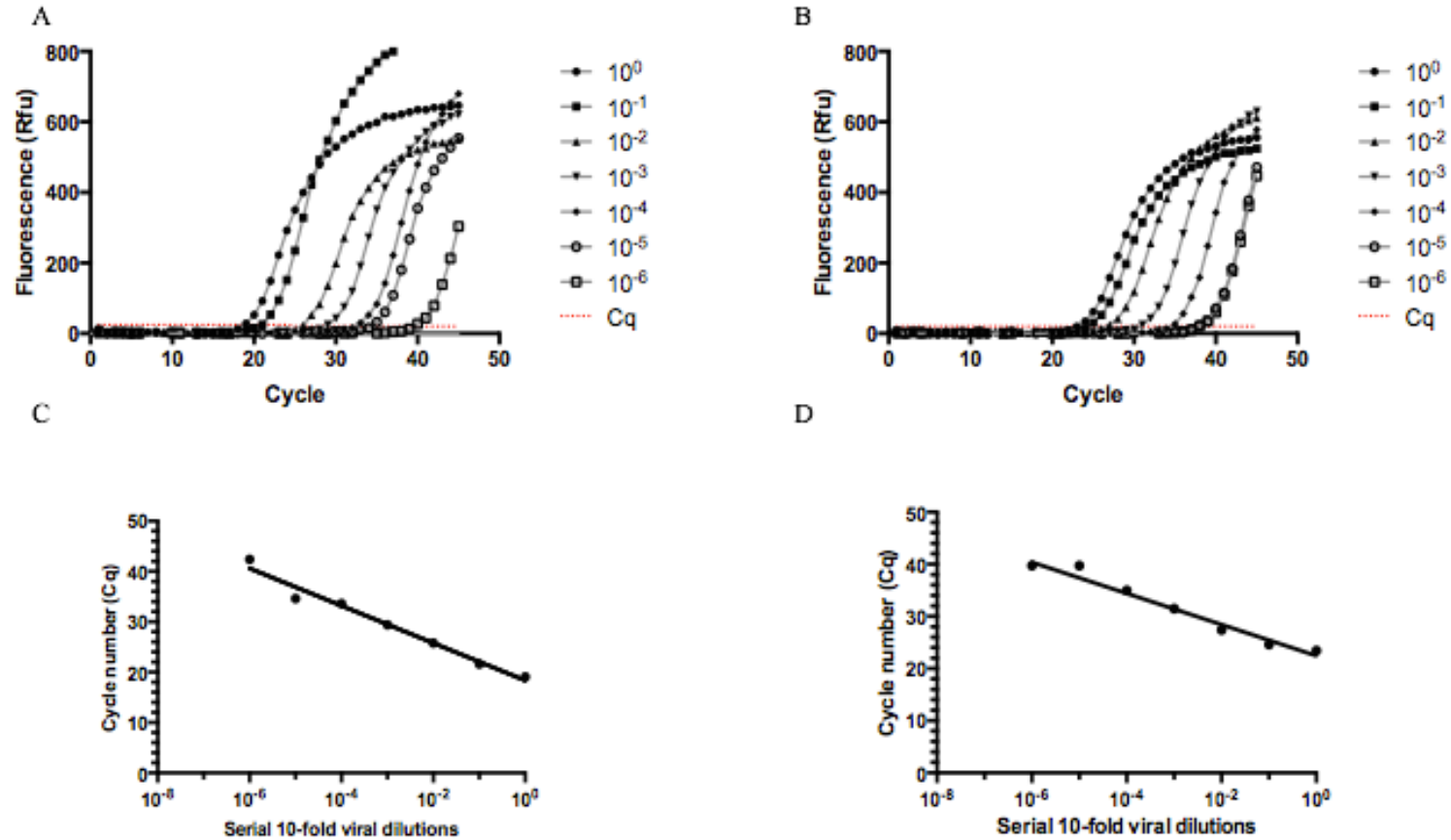
4.2.2 Relating viral titre to reverse transcriptase activity

In performing serial viral passages in the presence of increasing concentrations of C34-PEG₄-Chol, it was reasoned that a standardized inoculum of virus must be introduced from one passage to the next. Moreover, by detecting an increase in RT activity over the course of 7 days of viral culture in the presence of drug, HIV replication could be measured relative to day 0. PCR-enhanced RT assays (PERT) are sensitive tools for the detection of retroviruses which, through the addition of fluorescent probes, such as SYBR Green, may allow semi-quantitative analysis of a virus titre. They rely on intrinsic virion-associated RT acting on an extrinsic RNA target which is reverse-transcribed to DNA. After addition of a SYBR green fluorescent dye, the probe intercalates between bases pairs and fluoresces when bound to double stranded DNA. This produces a signal which is directly proportional to the amount of DNA detected. In turn, this is directly proportional to retrovirus-associated RT because RT concentration is directly associated with the overall retroviral. This signal is measured by the cycle of quantification (Cq) at which the fluorescence meets a minimum threshold. In this way, the assay can be used to perform a relative quantification of viral titre from one sample to another. Furthermore, by devising standard curves prepared from samples of known virus titres, absolute concentrations can be derived. The concentration of any unknown sample can then be determined by interpolating the Cq value of its PCR signal into a standard curve. Due to the efficiency with which the assay can be performed to quantify viral titres, the SG-PERT assay was chosen to provide a convenient, relative measurement of viral titre when passaged in cultures with C34-PEG₄-Chol. Prior to initiation of serial passages, a relationship was sought between viral titre, as determined by TCID₅₀/ml and RT activity as determined by the cycle of quantification (Cq) on the SG-PERT assay.

Serial 10-fold dilutions of BaL and NL4-3 virus stock were prepared and amplification curves generated via SG-PERT. As expected, a lower Cq value was obtained for lower 10-fold titrations indicating that higher concentrations of RT were present in more concentrated samples and, thus, fewer cycles were required to reverse transcribe the external RNA target and allow the fluorescence threshold to be reached when SYBR green was added. After deriving the Cq for each dilution, a standard curve of virus dilutions against the Cq was plotted. From this standard curve, viral titre could be semi-quantified for samples of unknown viral titre by determining the Cq of a viral culture and interpolated against the equivalent dilution on the standard curve. Since each reduction in the Cq value

represents a doubling of the titre, by undertaking two-fold dilutions of viral supernatant from cultures, a standardised inoculum of virus (equivalent to between 10^{-4} and 10^{-5} dilution of stock, Cq 34-36) could then be introduced into subsequent passages of virus when cultured with C34-PEG₄-Chol (as described in section 2.5). Over six 10-fold serial dilutions of both NL4-3 and BaL, a similar linear relationship was observed between Cq and dilutions, despite a substantial difference in the virus titres as calculated by Reed-Muench formulae and expressed by TCID₅₀/ml (**Figure 4.2 C and D**).

Figure 4.2 SG-PERT amplification and standard curves for serial 10-fold dilutions of NL4-3 and BaL



SG-PERT was used to measure RT concentrations as a surrogate marker for titres of NL4-3 and BaL calculated by BioRad software. **A)** Amplification curves obtained with serial 10-fold dilutions of NL4-3 stock. **B)** Amplification curves obtained with serial 10-fold dilutions of BaL stock **C)** Standard curve for NL4-3 serial 10-fold dilutions against Cq. Graph presented represents conglomeration of triplicate amplification curves. **D)** Standard curve for BaL serial 10-fold dilutions against Cq. Graph presented also represents conglomeration of triplicate amplification curves. The $TCID_{50}/mL$ for both NL4-3 and BaL lay between 10^{-4} and 10^{-5} dilutions.

4.2.3 Determining the IC₅₀ and IC₉₀ for C34-PEG₄-Chol

Pharmaceutical analyses of C34-PEG₄-Chol indicated that its IC₉₀ across a panel of laboratory viral clones lay between 0.05nM and 0.60nM (**Table 1.3.2**); to validate this result, viral entry into a receptive T-cell line in the presence of increasing concentrations of C34-PEG₄-Chol was assessed. TZM-bl cells stably express high levels of CD4, CCR5 and CXCR4 and contain reporter gene constructs, linking the HIV LTR promoter to luciferase; expression of the reporter gene is induced by the viral protein, Tat. Infection of TZM-bl cells with HIV results in production of Tat; in turn, there is activation of transcription from the HIV LTR, leading to expression of luciferase. The addition of a luciferase substrate allows luciferase activity to be measured by luminescence. Activity is directly proportional to the number of infectious viral particles gaining entry to the target TZM-bl cells.

The IC₅₀ and IC₉₀ was only determined for NL4-3. Serial 10-fold dilutions of NL4-3 stock were prepared from undiluted NL4-3 stock to 10⁻³. Aliquots of the laboratory strain, between 10⁻¹ and 10⁻³ dilutions were allowed to adsorb onto TZM-bl cells in triplicate on a 96-well plate. After incubation for 4 hours, virus supernatant was removed and replaced with culture medium containing C34-PEG₄-Chol in final concentrations ranging from 0-3.2 nM. After incubation for 72 hours, a lysis buffer was applied and luminescence measured on a luminometer. Luminescence, as measured in arbitrary units, was generated in triplicate for each dilution of virus across a range of drug doses from 0 to 3.2nM. The dose-response curve was generated by plotting the log₁₀ concentration of dose of C34-PEG₄-Chol against the the percentage inhibition in luminescence (**Table 4.3 and Table 4.4**), where 100% luminescence was generated in wells where no C34-PEG₄-Chol was present. IC₅₀ and IC₉₀ were then inferred from the dose-response curves, as plotted in **Figure 4.3**. Different IC₅₀ and IC₉₀ values were estimated, depending on the starting virus dilution. At the dilution 10⁻¹, the IC₅₀ was estimated as approximately 0.25nM whilst the IC₉₀ was estimated approximately 0.8nM; at the 10⁻² dilution, the IC₅₀ was estimated as 0.025nM whilst the IC₉₀ was estimated as 0.1 nM. At the 10⁻³ dilution, the IC₉₀ could not be inferred from the dose-response curve, however IC₅₀ was estimated as 0.035nM.

Table 4.3: Inhibition of virus entry into TZM-bl cells by C34-PEG₄-Chol

	Plate 1 Read 2 Gain 4000															
	0nM				0.05nM				0.1nM				0.2nM			
10⁻¹	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
Luminescence	400019	400019	400019	400019	400019	400019	400019	400019	400019	400019	400019	400019	253318	231354	223459	236043.7
% inhibition	0	0	0		0	0	0		0	0	0		36.7	42.2	44.1	
10⁻²	1	2	3	mean	1	2	3		1	2	3	mean	1	2	3	mean
Luminescence	373915	400019	395929	385944.5	106391	112438	125558	114795.7	73956	47278	30718	50650.7	27152	37419	23745	29438.7
% inhibition	3.1	-3.6	-2.6		72.4	70.9	67.5		80.8	92.0	92.0		93.0	90.3	93.8	
10⁻³	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
Luminescence	45706	42879	35146	41243.7	13309	20823	15053	16395	13336	10034	9296	10888.7	9906	8756	9927	9529.7
% inhibition	-10.8	-4.0	14.8		67.7	49.5	63.5		67.7	77.5	77.5		76.0	78.8	75.9	

Luminescence and percentage inhibition of viral entry into TZM-bl cells across dilutions from 10⁻¹ to 10⁻³ following incubation over 72 hours with increasing concentrations of C3.

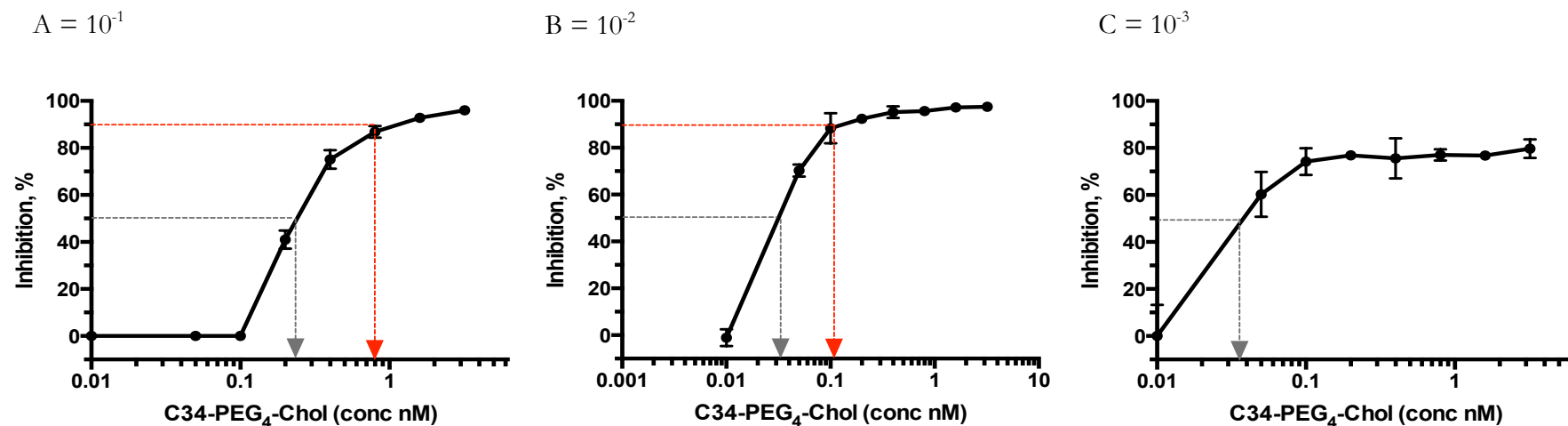
Percentage (%) inhibition was calculated using the formula:

$$\text{Percentage inhibition (\%)} = \frac{(\text{Luminescence in absence of drug}) - (\text{Luminescence in presence of drug})}{(\text{Luminescence in absence of drug})} \times 100\%$$

Table 4.4: Inhibition of virus entry into T₂M-bl cells by C34-PEG₄-Chol

	Plate 2 Read 2 Gain 4000															
	0.4nM				0.8nM				1.6nM				3.2nM			
10⁻¹	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
Luminescence	107421	81375	109697	99497.7	64034	47113	46174	52440.3	28142	26108	31623	28624.3	13005	14840	19963	15936
% inhibition	73.1	79.7	72.6	73.1	84.0	88.2	88.5		93.0	93.5	92.1		96.7	96.3	95.0	
10⁻²	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	Mean
Luminescence	11321	29602	15038	18653.7	17623	11786	21410	16939.7	9723	11328	11208	10753	10583	9140	9061	9594.7
% inhibition	97.1	92.3	96.1		95.4	96.9	94.5		97.5	97.1	97.1		97.3	97.6	97.7	
10⁻³	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
Luminescence	7704	14136	8334	10058	10433	9553	8471	9485.7	10269	9544	8915	9576	9626	8953	6518	8365.7
% inhibition	81.3	65.7	79.8		74.7	76.8	79.5		75.1	76.9	78.4		76.7	78.3	84.2	

Figure 4.3: Dose-response curve showing NL4-3 susceptibility to C34-PEG₄-Chol



Dose-response curve of percentage (%) inhibition in luminescence against log concentration of C34-PEG₄-Chol (conc nM). NL4-3 was incubated in triplicate with TZM-bl cells in the presence of C34-PEG₄-Chol across concentrations ranging from 0 to 6.4 nM. The IC_{50} (dashed grey arrow) and IC_{90} (dashed red arrow) were inferred from the graph by interpolation between the drug concentrations tested *in vitro*. At the dilution 10^{-1} , the IC_{50} was estimated as approximately 0.25nM, while the IC_{90} was estimated approximately 0.8nM. At the 10^{-2} dilution, the IC_{50} was estimated as 0.035nM while the IC_{90} was estimated as 0.1 nM. At the 10^{-3} dilution, the IC_{90} could not be inferred from the dose-reponse curve, however IC_{50} was estimated as 0.035nM.

4.2.4 Selection of viruses resistant to C34-PEG₄-Chol

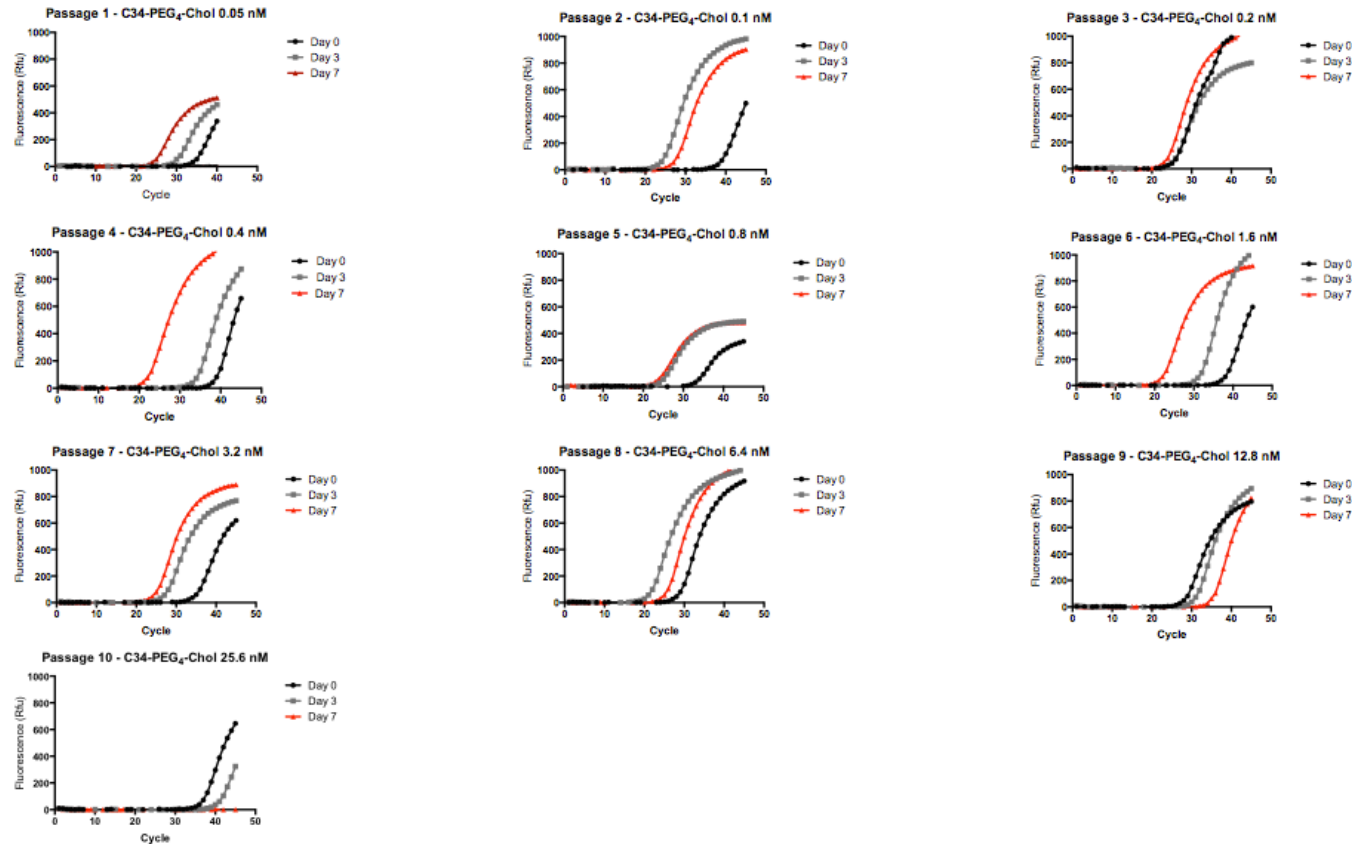
HIV isolates resistant to C34-PEG₄-Chol were derived by repeated passage of BaL and NL4-3 in the PM-1 cell line with increasing concentrations of the drug. For both NL4-3 and BaL, cultures inoculated with virus diluted to a Cq of 32-34 (equivalent to 10⁻⁴ dilution of viral stock on the standard curve or 20 pfu/mL) and incubated for several hours with PM-1 cells. Cultures were started at a concentration of C34-PEG₄-Chol of 0.05nM (equivalent to the IC₅₀ of wild-type NL4-3) and doubled at each passage. NL4-3 and BaL supernatants were collected on day 7 of each passage and again diluted to a Cq between 32-34. Discussions with the peptide manufacturer indicated that although the peptide was only considered to be stable for up to 24 hours at room temperature after reconstitution, the lower pH generated by metabolically active cells in culture medium would enhance stability of the peptide for a longer period; as such, every 72 hours, the medium was supplemented with medium containing the same concentration of drug as introduced at the start of the passage. Viral growth was monitored by SG-PERT from supernatant collected every 72 to 84 hours and by the appearance of a cytopathic effect (CPE) in PM-1 cells. At the end of the passage, SG-PERT was undertaken on supernatants collected over the week to assess viral growth, as indicated by change in RT activity between day 0 and 7; in addition, the viral titre from supernatant on the final day of culture could be approximated from the standard curves according to the Cq value (**Figure 4.2**). Viral supernatants from day 7 could then be diluted to a Cq between 34-34 and introduced into the next passage with a doubling of drug concentration on the culture medium. At lower drug concentrations (0.05-0.6nM), CPE were observed following 48-72 hours of culture but less readily observed above concentrations of 1.6nM.

Viral passages of BaL were continued until a maximum concentration of C34-PEG₄-Chol of 25.6 nM (**Figure 4.4**). Review of change in RT activity between day 0 and day 7 indicated that up to a drug concentration of 6.4 nM (passage 8) viral growth was observed as indicated by a reduction in the Cq value between day 0 and day 7. At concentrations of 12.8 nM and 25.6 nM, growth of BaL was completely inhibited as indicated by a decline in RT activity between day 0 and 7 (with an increase in Cq value) and no CPE were observed in cell cultures (**Figure 4.4**). With continued viral growth up to 6.4 nM (between 10-128 times the IC₉₀ of C34-PEG₄-Chol, based on IC₉₀ 0.05-0.60nM) it was assumed that a drug resistant viral population was emerging. With cessation of viral growth beyond 6.4 nM, it could be assumed that viral growth was either completely inhibited by higher drug

concentrations, despite the presence of resistance associated mutations or, that a viral population had emerged which had become replication incompetent as a consequence of resistance-associated mutations.

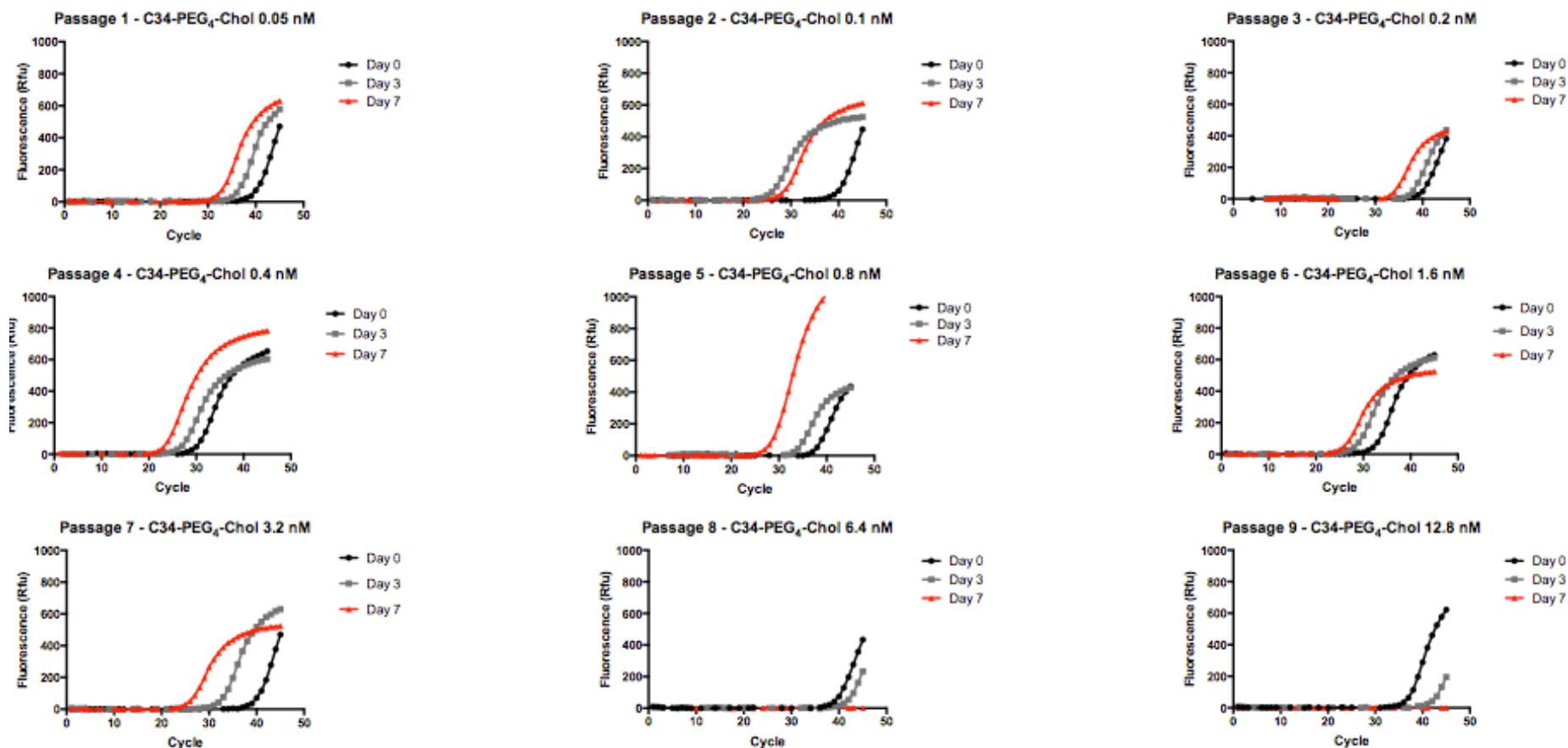
Similarly, viral growth as indicated by an increase in RT activity was observed up to a concentration of C34-PEG₄-Chol of 3.2 nM; beyond this, there was a decline in RT activity noted with doubling concentrations of drug (**Figure 4.5**).

Figure 4.4: Change in RT activity of BaL over 7 days with doubling concentrations of C34-PEG₄-Chol



SG-PERT growth curves demonstrating the change in RT activity as determined by the cycle of quantification measured on days 0, 3 and 7 after initiating BaL cultures. RT activity increases between days 0 and 7 in passages 1 to 8, indicating ongoing viral turnover in the presence of the stated concentration of drug. Within passages 9 and 10, there is a decline in RT activity between days 0 and 7 indicating a lack of viral growth.

Figure 4.5: Change in RT activity of NL4-3 over 7 days with doubling concentrations of C34-PEG₄-Chol



SG-PERT growth curves demonstrating the change in RT activity as determined by the cycle of quantification measured on days 0, 3 and 7 after initiating NL4-3 cultures. RT activity increases between days 0 and 7 in passages 1 to 7, indicating ongoing viral turnover in the presence of the stated concentration of drug. Within passages 8 and 10, there is no increase in RT activity over 7 days of culture, indicating a lack of viral growth.

4.2.5 Reverse transcription and amplification of PCR products after serial passages of virus

Following the final passage, NL4-3 and BaL RNA were extracted, reverse transcribed and amplified as described in section 2.1.8. After second round PCR, products underwent agarose gel electrophoresis to confirm amplification prior to direct sequence analysis (**Figure 4.6**). There was reduced signal under ultraviolet light following gel electrophoresis in passage 10 for both NL4-3 and BaL, indicating reduced viral titre.

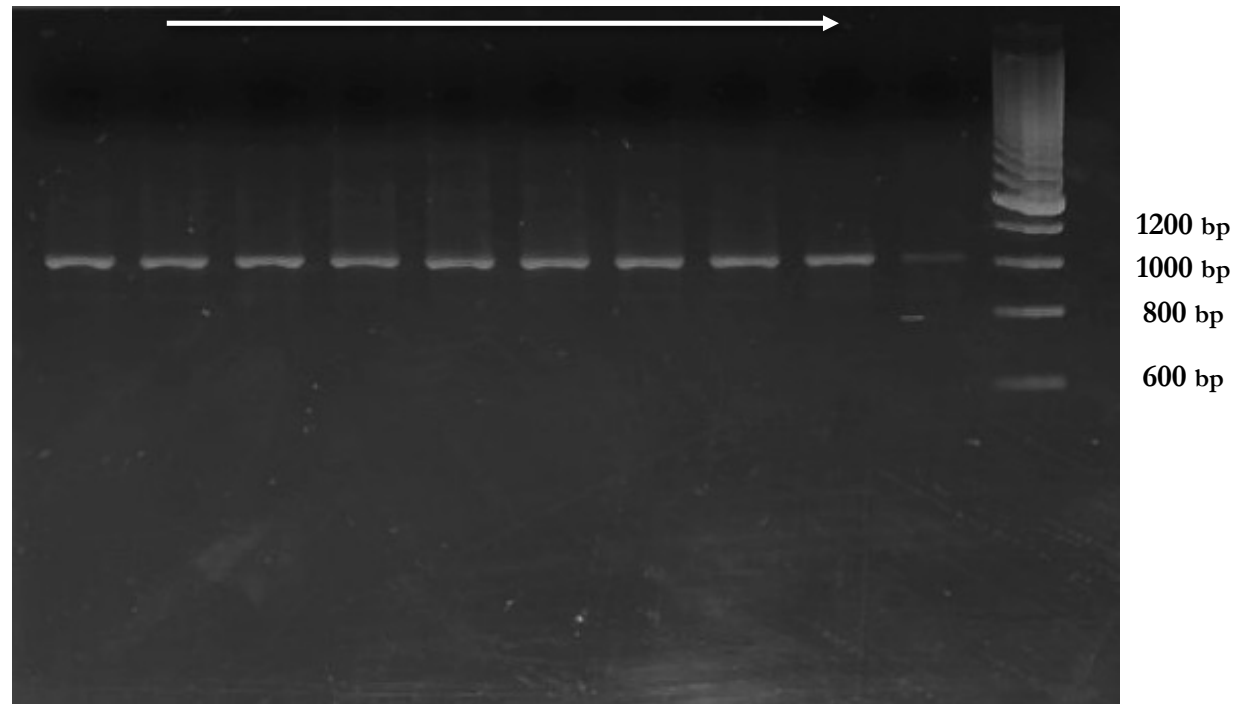
4.2.6 Sequence analysis of HIV-1 gp41 following serial passages

Direct sequence analysis of the ectodomain of gp41 from serial passages of NL4-3 and BaL was achieved using gp41 primers 3, 4, 5 and 6, as described in section 2.1.8.7. Despite serial passages showing viral replication up to drug concentrations of 3.2 nM, analysis of the gp41 ectodomain of NL4-3 failed to demonstrate the evolution of any mutations in the HR-1 region (**Figure 4.7**). After nine passages of BaL over a 10-week period, a virus population emerged which grew in drug concentrations of 6.4 nM; beyond this concentration (at 12.8 nM and 25.6 nM), there was a decline in RT activity between days 0 and 7, indicating complete inhibition of viral growth.

With these initial passages, mutations emerged within the HR-2 subdomain, namely at position 148 (glutamic acid to lysine) emerging at 6.4nM, position 136 (glutamic acid to glycine) at 12.8nM and unknown amino acid substitutions at position 71, indicating emergence of a resistant viral strain between 21.3 and 42.6-fold the IC_{90} of the drug (**Figure 4.8**). To further characterise the presence of mutations within the HR-1 domain of gp41, repeat passages of BaL were undertaken. BaL cultured at 3.2 nM was subjected to a further prolonged culture for 1-week at 3.0 nM. Cultures were observed for the appearance of CPE over a 1-week period before passaging viral supernatant at a higher concentration of drug. In order to avoid overshooting the zone of mutant selection, drug concentrations were escalated at narrower increments (0.5nM and 1.0nM). Viral cultures were continued until no CPE were observed in cells after an incubation period of 1 week indicating either complete inhibition of viral replication by the concentration of drug present or due to the emergence of mutations rendering the virus replication-incompetent. Cultures were stopped at a concentration of C34-PEG₄-Chol of 9.5 nM, at which point no CPE were observed after 1-week. Repeat direct sequence analysis again revealed

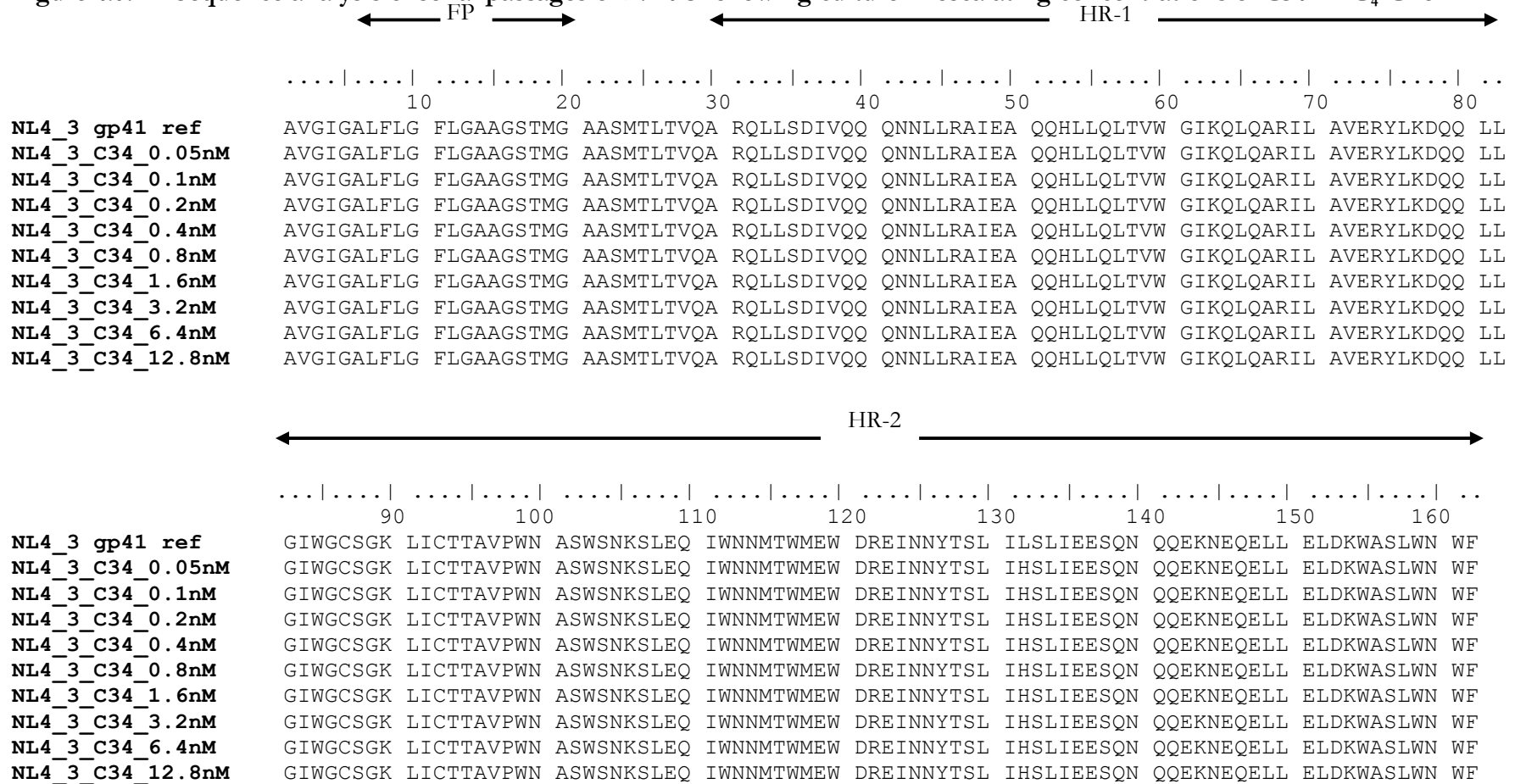
emergence of E136G, E148K and, in addition, characterized the mutation at position 71 (alanine to threonine) within the HR-1 region (**Figure 4.9**).

Figure 4.6: Gel electrophoresis demonstrating amplification of BaL following serial passages



Gel electrophoresis demonstrating amplification of second round PCR products of BaL (passages 1-10) aligned against molecular weight marker ladder; passage 10 shows a fainter band due to a lower virus titre at Day 7 and corresponding with the reduced viral replication observed on SG-PERT in Figure 3.4. Note, molecular weight markers below 600 base pairs (bp) are not visible on photograph.

Figure 4.7: Sequence analysis of serial passages of NL4-3 following culture in escalating concentrations of C34-PEG₄-Chol



Amino acid sequence of the gp41 ectodomain of NL4-3: serial passages with doubling of the concentration of C34-PEG₄-Chol did not result in the evolution of any mutations on sequence analysis despite evidence of increase in RT activity up to a concentration of C34-PEG₄-Chol of 3.2 nM.

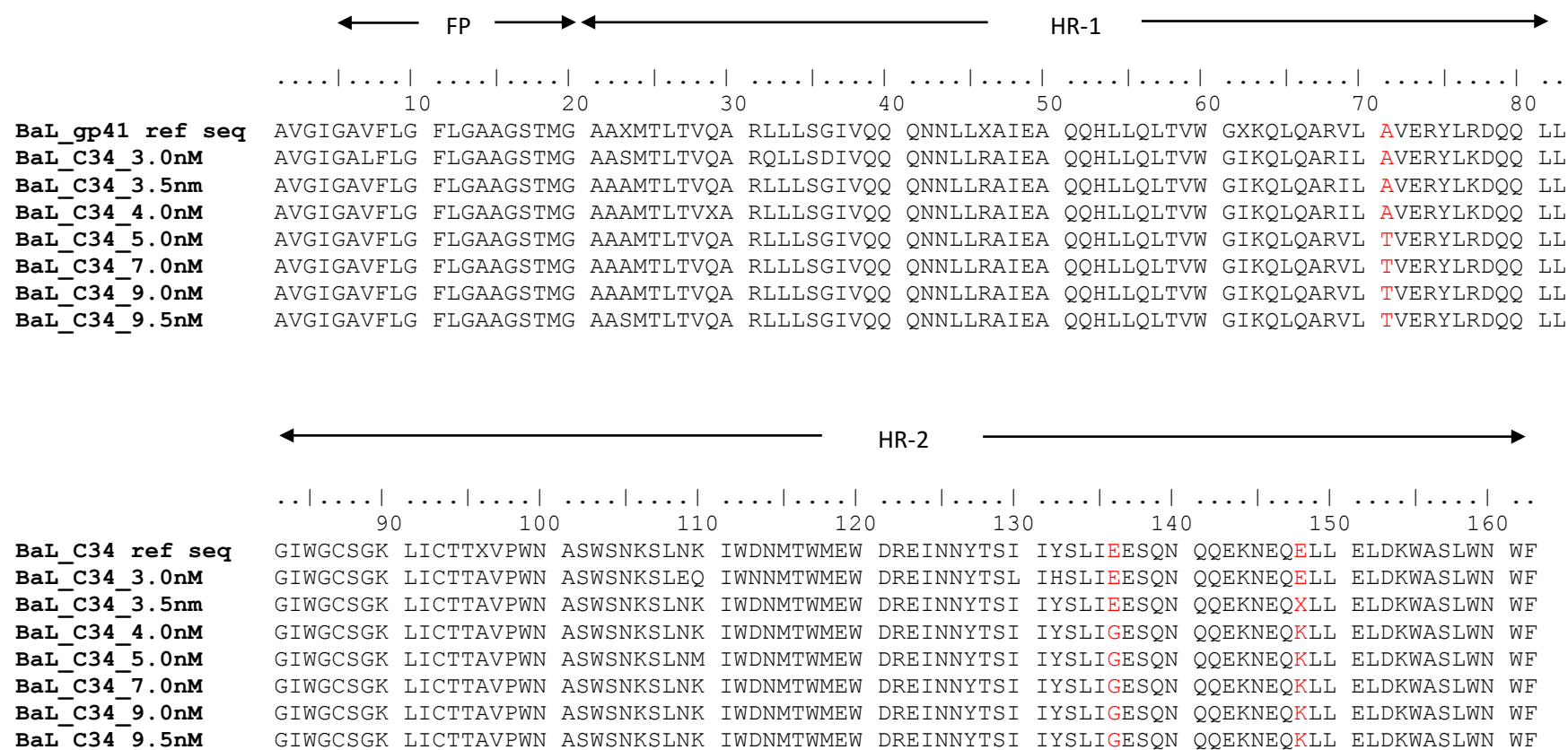
Figure 4.8: Sequence analysis of serial passages of BaL following culture in escalating concentrations of C34-PEG₄-Chol

		FP										HR-1											
																							
		10 20 30 40 50 60 70 80																					
BaL_gp41	ref seq	AVGIGAVFLG	FLGAAGSTMG	AAXMTLTVQA	RLLLSGIVQQ	QNNLLXAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	0.05nM	AVGIGAVFLG	FLGAAGSTMG	AAXMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	0.10nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	0.20nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	0.40nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	0.80nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	1.60nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	3.20nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	6.40nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GXKQLQARVL	AVERYLRDQQ	LL													
BaL_C34	12.80nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARVL	XVERYLRDQQ	LL													
BaL_C34	25.60nM	AVGIGAVFLG	FLGAAGSTMG	AAAMTLTVQA	RLLLSGIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARVL	XVERYLRDQQ	LL													

		HR-2											
													
		90 100 110 120 130 140 150 160											
BaL_C34	ref seq	GIWGCSGK	LICTTXVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF			
BaL_C34	0.05nM	GIWGCSGK	LICTTXVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF			
BaL_C34	0.10nM	GIWGCSGK	LICTTXVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF			
BaL_C34	0.20nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLEK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF			
BaL_C34	0.40nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF			
BaL_C34	0.80nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF			
BaL_C34	1.60nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQXLL	ELDKWASLWN	WF			
BaL_C34	3.20nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIEESQN	QQEKNEQXLL	ELDKWASLWN	WF			
BaL_C34	6.40nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINXYTSI	IYSLIEESQN	QQEKNEQKLL	ELDKWASLWN	WF			
BaL_C34	12.80nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIGESQN	QQEKNEQKLL	ELDKWASLWN	WF			
BaL_C34	25.60nM	GIWGCSGK	LICTTAVPWN	ASWSNKSLNK	IWDNMTWMEW	DREINNYTSI	IYSLIGESQN	QQEKNEQKLL	ELDKWASLWN	WF			

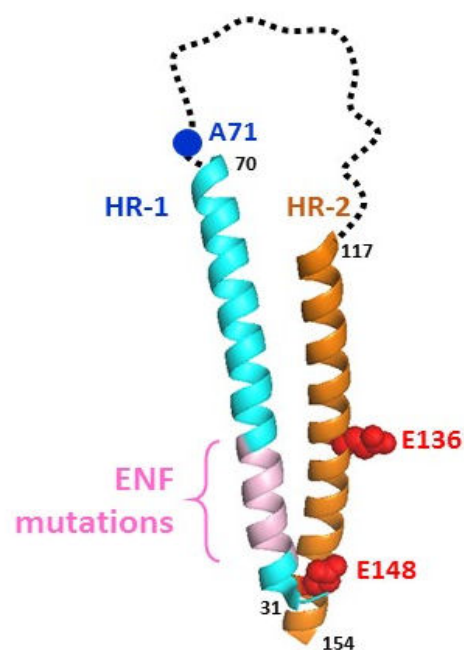
Amino acid sequence of the gp41 ectodomain of BaL: BaL reference sequence with evolution of amino acid substitutions across the HR-1 (unknown mutation at position 71) and HR-2 domains (E136G and E148K) following successive cultures in increasing concentrations of C34-PEG₄-Chol.

Figure 4.9: Sequence analysis of repeated serial passages of BaL following repeat culture in escalating concentrations of C34-PEG₄-Chol



Repeated cultures of BaL with narrower increments in drug concentration between passages: BaL reference sequence with evolution of amino acid substitutions across the HR-1 (A71T) and HR-2 domains (E136G and E148K).

Figure 4.10: 3-Dimensional cartoon of gp41 with mutations arising with C34-PEG₄-Chol mapped



The mutations arising upon treatment with C34-PEG₄-Chol (red and blue) are mapped onto the post-fusion crystal structure of gp41. Residue A71 is just upstream of the last residue present in the structure, and is depicted as a blue sphere on the cartoon representing the connection between HR-1 and HR-2. The location of mutations arising upon treatment with enfuvirtide (ENF) are represented in pink.

4.3 Discussion

The above results have proven the hypothesis that in the presence of residual HIV viral replication, drug pressure would result in the selection of mutations in the HR-1 region of gp41. Serial passages of BaL in increasing concentrations of C34-PEG₄-Chol selected a single point mutation at position 71 in the HR-1 domain of gp41 lying immediately adjacent to the PD of gp41. Simultaneously, presumed compensatory mutations emerged within the HR-2 domain at locations 136 and 148 (**Figures 4.8 and 4.9**). The emergence of the primary mutation, A71T within the PD of HR-1 contrasts with previous studies examining the emergence of resistance mutations with the native C34 molecule which selected mutations at positions 33 (L33S, leucine to serine) and 38 (V38E, valine to glutamic acid) occurring within the LD [146].

Although the mutation is located upstream from mutations previously described with the native C34 molecule, A71T has previously been described with *in vitro* passage experiments with SFT (which contains the C34 sequence), albeit in combination with other mutations. Within this study, although A71T invoked only a two-fold reduction in sensitivity to SFT, when combined with other mutations within HR-1 and HR-2, resistance between 150 and 600-fold was observed [147].

While the significance of the A71T mutation is unclear, it should be noted that locus 71 lies immediately adjacent to the predicted zone of interaction of C34 on the PBD (**Figure 4.11**). Given the high potency of C34 is thought to be resulting from the interaction between the PBD incorporated into its structure with the PD in HR-1, it would seem logical to assume that maximal resistance would be achieved through selection of mutations within its binding site, the PBD subdomain of HR-1. Indeed, the importance of the PBD in inducing resistance to C34 was demonstrated however through construction of pseudoviruses containing point mutations at position 67 within the PBD which conferred resistance of between 100- and 243-fold to the native C34 [76]. To date, however, various studies examining evolution of resistance-associated mutations with C34 and related compounds have only seldomly selected mutations within this region [76, 148]. Consequently, it has been postulated that the PBD is highly conserved but mutations occurring immediately adjacent to the PBD may be of added significance.

Secondary mutations are also well described; perhaps best known, the S138A mutation occurring in conjunction with the N43D mutation following exposure to enfuvirtide has been shown to enhance resistance to enfuvirtide by over threefold than that conferred by the HR-1 mutation N43D alone [149]. This additive effect is thought to result from restoration of impaired replication kinetics of enfuvirtide resistant variants that contain primary mutations in the HR-1 region of gp41.

Both the E136G and E148K mutations have been selected in studies examining resistance to the third generation HR-2-based FI, T2635. Although their individual effect on drug resistance has not been studied, E136G and E148K have been estimated to contribute to 63-fold resistance and 14.4 fold resistance, respectively to T2635 when occurring in combination with other mutations [112].

A number of limitations exist in the present study. In the first instance, dual cultures were set up with NL4-3 and BaL; this contrasts with studies conducted by other groups who set up multiple parallel cultures to evaluate the evolution of mutations, for example, Eggink et al set up 34 evolution cultures when investigating the evolution of mutations with T2635 [112]. Moreover, in these studies the cell line SupT1 was used along with the molecular clone HIV_{LAI} while growth was monitored with a p24 capsid assay and use of these methods may have proven more fruitful [112, 150]. Although evolution of resistance-associated mutations occurs under selective drug pressure, it is recognized that not all mutations will emerge in a single culture with repeat passages in the presence of drug and that there is likely to be more than one mutation within the HR-1 subdomain contributing to resistance to C34-PEG₄-Chol. Moreover, while other mutations within the HR-1 subdomain may, indeed, have emerged under drug pressure, with Sanger sequencing, only viral populations which represent $\geq 20\%$ of the viral quasi species will be identified and as such, these minority variants may not have been identified [151]. The detection of other mutations may also have been limited by a rapid ascent in drug concentrations from one passage to the next (**Figure 4.12**); in doing so, the zone of mutant selection may have been traversed too rapidly, thus limiting the opportunity for resistance-associated mutations to emerge within the quasi species. While attempts were made to address this potential limitation by re-culturing BaL from a drug concentration of 3.2 nM at smaller increments, by using a viral culture which had already been exposed to C34-PEG₄-Chol, the resistance pathways within the culture were likely to have already established and so, mutations other

than A71T may be less likely to emerge. To overcome these limitations, it may have been preferable to expand the number of viral cultures undertaken, increment passages with narrower concentrations of C34-PEG₄-Chol and/or to use newer methodologies to identify minority variants such as ultra-deep sequencing. Unfortunately, optimisation of methods was limited by both time and the expense of development of an ultra-deep sequencing assay.

Titration of equal inocula of virus into successive cultures presented challenges. A TCID₅₀ infectivity assay was used to quantify replication-competent infectious virus titres in culture supernatants. This infectivity assay was used due its simplicity in performing within C8166 cells although other assays, such as a plaque forming assay, could have been used as an alternative. The importance of these assays lies in their ability to determine the titres of viable, infectious virions as opposed to more modern techniques, such as RT assays (measure relative intrinsic RT activity) or capsid p24 assays (which measure protein derived from virus) both of which may provide a surrogate measure of viral titres but cannot discern between viable infectious viral particles and their derivatives. Additionally, capsid p24 assays rely on enzyme-linked immunosorbent assay (ELISA) and consequently have a limited linear range. The value of infectivity assays is highlighted when one considers that the high frequency of mutations in the HIV genome introduced during reverse transcription can render a large proportion of viral particles defective, replication incompetent or dead. For example, it is estimated that only around 1% of viral particles in an HIV infected individual are actually infectious. Of this 1%, many infectious virus particles will vary in fitness due to variations in the sequence substitutions or due to inefficient assembly or new virions [152]. Hence, an assay which accurately determines the infectious viral titre, as opposed to the absolute viral titre, is critical. These assays however are cumbersome and specifically, the TCID₅₀ arguably suffers from difficulty in tangibly understanding what the absolute viral titre is thus presenting difficulties with interpretation. This may have been offset by use of multiplicity of infection or MoI as an alternative measure of infectious viral titre. MoI calculates the ratio of infectious agents (i.e. virus) to infection targets (i.e. cell) and can be expressed using any quantitative measure of virus titre, for example particles/cell, TCID₅₀/cell or plaque forming units (pfu)/cell. MoI can be approximated from TCID₅₀ as a multiple of 0.69 derived through Poisson distribution. Although MoI may have been a preferable measure over the use of TCID₅₀, there are concerns that this relationship may not hold even for the same virus and cell

combinations, as two assays may be set up with subtle differences and viral infectivity may be sensitive to various factors such as cell age, overlay media and as such using MoI to calculate inocula was not done.

Attempts were made to relate the $TCID_{50}$ to RT using a PERT assay. In doing so, it was proposed, that should a linear relationship be established through standard curves, rapid quantification of viral titres could be determined in cell-free supernatants and allow a more rapid turnover of passages. Defining this relationship, however was limited by difficulties in obtaining uniform standard curves with serial 10-fold dilutions and is thus likely to have impacted adversely in the ability to transfer equal titres from one culture to the next, which in turn may have impacted on culture kinetics and what mutations emerged. Moreover, although it is assumed that one RT unit exists per virion and thus that RT activity was directly proportional to viral titre, it is plausible that RT activity varies at different viral concentrations thus could give spurious measurements of viral titres. SYBR-Green PERT assays, however, have previously been shown to correlate well with transducing and infectious virion particles of HIV in replication competent preparations [153].

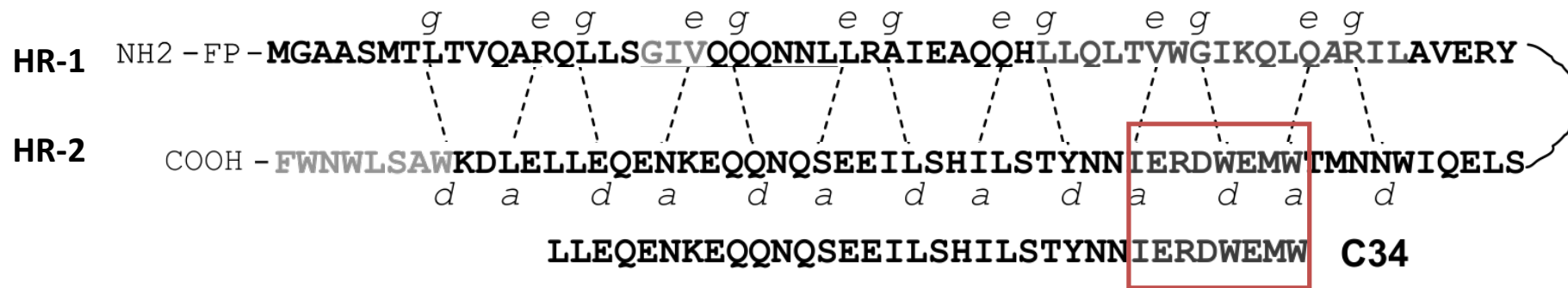
As discussed, surrogate assays such as p24 capsid, the endogenous RT activity, and amount of viral genomic RNA in virus particles are now commonly used in research laboratories but have not completely superseded the use of infectivity assays. In a recent literature review, approximately 50% of studies comparing the replication efficiencies of different HIV isolates or mutants continued to measure virus titres by $TCID_{50}$ assays, whilst 45% used p24 antigen capture assays, and only 5% used RT assays [154]. Direct comparison of these assays and actual infectious HIV titres from $TCID_{50}$ assays has only been performed with the most conserved laboratory strains. A recent study however indicated that endogenous RT activity and not p24 content or viral RNA load, was a superior measure of infectious HIV titre in cell-free supernatants when analysed against $TCID_{50}$. Various HIV subtypes did not appear to affect the function or activity of the RT enzyme in this endogenous assay but did affect the detection of p24 capsid [154]. Nonetheless, within the results presented above, it was noted that, despite a significant difference in the viral titres between the stock viruses of NL4-3 and BaL, that a similar linear relationship existed between Cq and dilutions. This may suggest that different laboratory strains of HIV may produce RT with different efficiencies. Indeed, this seems likely given 15 to 30% diversity in nucleotide sequence between HIV-1 isolates from different group M subtypes which

may in turn influence RT structure and its efficiency and indeed variability in the efficiency of reverse transcription is recognized amongst different viral isolates [154, 155].

In undertaking serial passages, virus supernatant was allowed to adsorb onto cells for up to 4 hours before replacement with culture medium containing C34-PEG₄-Chol. This approach was used in order to follow similar methodologies employed during experiments by investigators of other FIs. Arguably, however, a mix of viral supernatant and cell culture medium containing C34-PEG₄-Chol at the concentration under investigation should have been added concomitantly; the rationale for this lies behind the fact that viral entry normal occurs in receptive cell lines within about 30 minutes. By allowing viral supernatants to adsorb for a number of hours before the addition of drug however, this may not have allowed viral particles containing mutations to C34-PEG₄-Chol to be selected for entry into receptive cells and as such may have limited the success of the experiments.

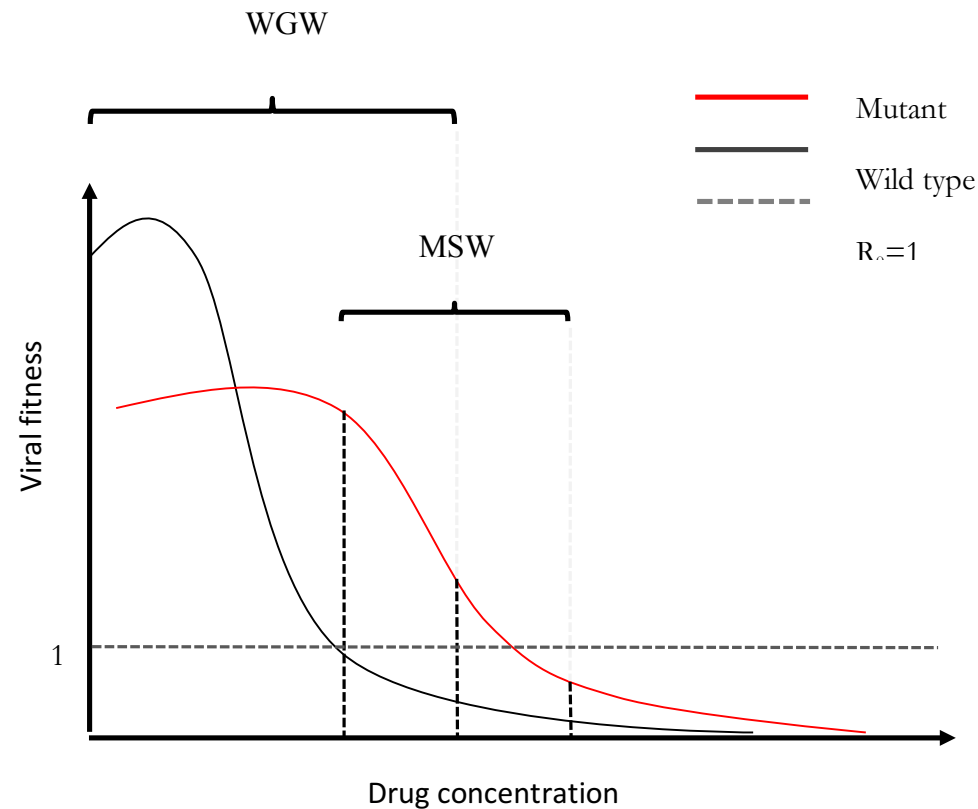
Finally, it is arguable that cloning with site-directed mutagenesis to introduce mutations into the HR-1 domain could have been used from the outset to determine the effect of various mutations on the susceptibility of the virus to C34-PEG₄-Chol. This of course may have answered the question of which areas of the HR-1 domain are critical to developing resistance but would not have helped to establish which mutations are likely to emerge in the presence of drug pressure, a critical question in clinical practice. Use of next generation sequencing (NGS) has thus far not entered widespread clinical use in the UK but undoubtedly will prove a useful adjunct in clinical management in coming years. Unfortunately, the use of NGS could not be employed within the current work owing to time and financial constraints. It is probable that while other mutations were selected during *in vitro* passage work, these were not sequenced due to their occurrence below the 20% cut-off level for detection by Sanger sequencing. It is likely that had a NGS library been developed, mutant variants at levels of 5% or below could have been detected and thus allowed the full array of mutations to be characterized, and indeed, numerous studies have demonstrated the ability of NGS to detect mutant variants between 0.1-1% of the total quasi species [156-159].

Figure 4.11: gp41 in hairpin configuration showing interactions between HR-1 and HR-2 and alignment of the C34 sequence



Interaction between HR-1 and HR-2; the letters “a-g” represent the positions of the corresponding residues in the helical wheel of gp41. The alignment of C34 is shown against the HR-2 domain with the PBD highlighted within the red square. *Adapted from: Lu et al. Biochim Biophys Acta. 2012 Dec;1818(12):2950-7*

Figure 4.12: Drug concentrations determine the relative fitness of the wild-type virus and a mutant resistant virus



The fitness of wild-type virus decreases with increasing drug concentrations; a drug resistant virus is less fit than wild-type virus at low drug concentrations and more fit at high drug concentrations. The mutant selection window (MSW) is the range of concentrations at which a resistant virus, if present, will grow faster than the wild type $R_0 > 1$. The wild-type growth window (WGW) is the range of drug concentrations where wild-type has $R_0 > 1$ leading to treatment failure without resistance. *Adapted from: Rosenbloom DI et al. Nat Med. 2012 Sep;18(9):1378-85*

Chapter 5

Phenotypic effects of gp41 mutations on
susceptibility to C34-PEG₄-Chol

5.1 Introduction

Resistance-associated mutations are independently associated with poor clinical outcomes in patients receiving ART. Determining the effects on drug susceptibility of a given amino acid substitution is, therefore, of paramount importance to clinical decisions when a new drug is introduced. As described, the process of identifying the mutations associated with resistance to a given ARV drug depends on 1) sequencing of the virus derived from *in vitro* passage when virus is cultured with drug and 2) from clinical samples of HIV-positive patients exposed to a drug. The phenotypic effects of mutations arising during passage of virus in the presence of drug are established through site-directed mutagenesis experiments in which resistance-associated mutations are introduced into the wild-type virus. The effects of the mutation are then tested by culture of the recombinant virus in the presence of the drug to determine if the mutation diminishes, preserves or enhances the replicative capacity of the virus when the drug is present. Mutations identified by this process acquire widespread acceptance as the predominant mutations responsible for drug resistance, and are referred to as ‘canonical’ resistance mutations.

Due to its interaction with the PD residues, 54-66, of gp41, it has been hypothesized that maximum resistance to C34 would be caused by amino acid substitutions within this region of HR-1. Indeed, logically, as these are the critical residues of interaction driving the antiviral effects of C34, it follows that mutations within this region should drive resistance to C34. Up to this point however, no *in vitro* studies with the C34 lead molecule have shown the emergence of mutations within the PD residues 54-70, leading to the hypothesis that the PD is too conserved for mutations to be selected.

To circumvent this problem, site-directed mutagenesis has been used to introduce arbitrarily mutations within the PD. Notably, Lu *et al* undertook site-directed mutagenesis to introduce alanine to glycine, aspartic acid and serine substitutions at residue 67 (A67G/D/S) which rendered the virus resistant to C34 by 60-, 100- and 244-fold, respectively compared with the native virus. Of note however, is that these isolates remained sensitive to enfuvirtide [76]. Thus, it has been suggested that that C34 and enfuvirtide inhibit viral entry by interacting with different target sites. Specifically, it has been proposed that enfuvirtide inhibits HIV entry by binding to the N-terminal region of the HR-1 domain and the lipid membrane of the target cell, while C34 blocks viral entry by interacting with the C-terminal region of HR-1 thus blocking viral gp41 6-HB

formation. By contrast, previous studies examining the *in vitro* evolution of resistance to C34 also failed to show emergence of mutations within the PD and, in fact, selected mutations within the LD at similar residues to that observed with enfuvirtide, most notably V38E mutation (conferring >1000-fold resistance to C34) and a new, previously undocumented mutation, L33S [146]. Moreover, *in vitro* passage experiments with T2635 and SFT could not select resistance-associated mutations in the gp41 pocket region, but rather at sites upstream from the PD adjacent to and within the LD. The finding of the A71T mutation with *in vitro* passage of BaL with C34-PEG₄-Chol, therefore, presents the interesting potential of identifying a resistance-associated mutation with a direct effect on susceptibility to C34 and which is immediately adjacent to the highly conserved PD.

The occurrence of accessory mutations in the HR-2 subdomain with use of HR-2-based FIs and their effects on drug susceptibility have already been recognised [160-163]. Most likely, their presence restores viral fitness and replicative capacity through improvements in viral fusion kinetics. It has been hypothesized that mutations within the HR-1 subdomain may delay fusion in the step after co-receptor engagement [163]. With this slowing in viral-cell fusion, it has been postulated that virus is rendered more susceptible to neutralisation antibodies that can bind to the membrane-proximal region of gp41 when it is exposed. The presence of accessory mutations within HR-2 may restore the kinetics of interaction between the HR-1 and HR-2 subdomains to form the 6-HB, thus mitigating against the vulnerability of the membrane-proximal region of gp41 to neutralizing antibodies.

Hypothesis

Mutations occurring in the presence of C34-PEG₄-Chol will confer reduced susceptibility of HIV to the drug.

Aims

Based on this discussion, the aims of the following chapter were:

1. to undertake site-directed mutagenesis to introduce C34-associated mutations (L33S and V38E) and mutations selected with C34-PEG₄-Chol (A71T, E136G and E148K) into gp41.
2. to subclone mutated gp41 into pNL4-3 (wild-type) plasmid.
3. to transfect mutated pNL4-3 plasmids to create virus stock of mutated NL4-3.

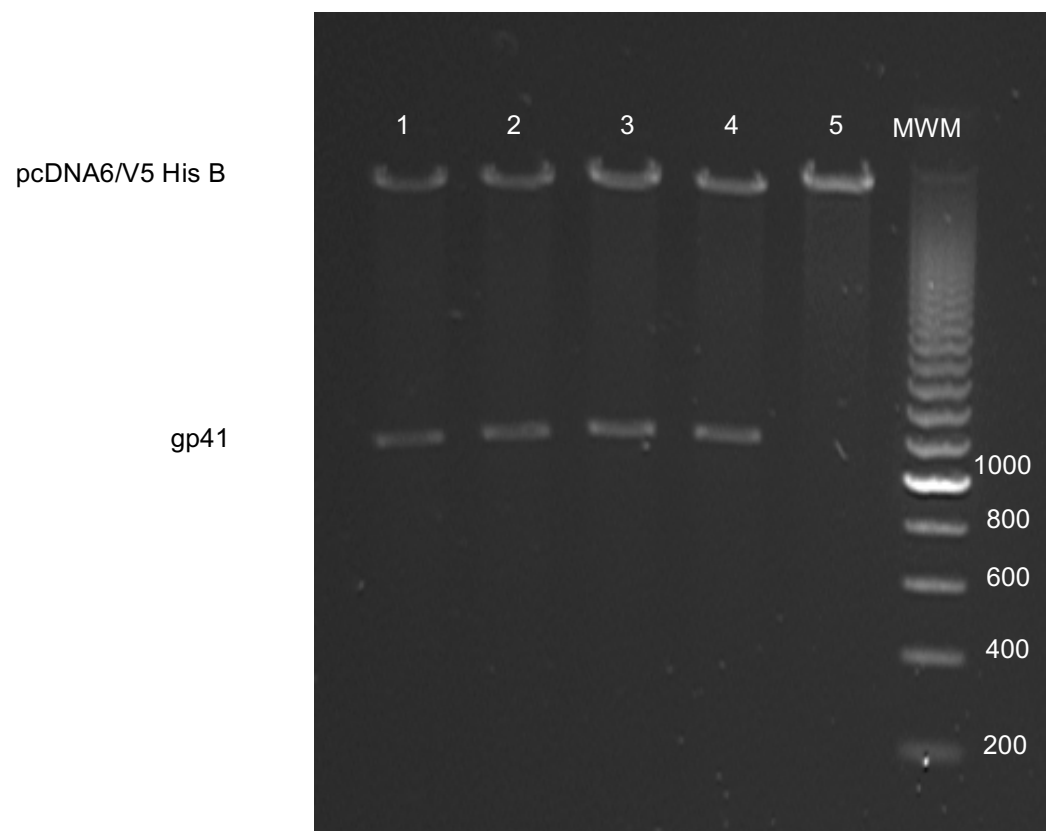
4. to determine the effect of the individual mutations on the susceptibility of NL4-3 to C34-PEG₄-Chol.

5.2 Results

5.2.1 Construction of recombinant plasmid, pcDNA6/V5-HisB_gp41

Construction of the double-stranded DNA template for site-directed mutagenesis was performed as described in section 2.1.9. The plasmid, pcDNA6/V5-His B, was chosen as the template into which the gp41 fragment was introduced. Both pcDNA6/V5-His B and pNL4-3 contain the endonuclease restriction enzyme sites, *Nhe* I and *Bam* HI. The enzymes were used to extract a fragment of gp41 from pNL4-3 corresponding to nucleotides 7250 to 8465 and, concurrently, to linearise pcDNA6/V5-His B. Crucially, the fragment excluded the gp41 nucleotide residues from 8466 to 8795, meaning that mutations located upstream from 8465 were omitted from the recombinant plasmid, pcDNA6/V5-HisB_gp41. In addition, the fragment contained residues of gp120. Following the ligation reaction between the gp41/gp120 fragment and pcDNA6/V5-HisB, creation of the recombinant plasmid was confirmed via a small-scale digest of pcDNA6/V5-HisB_gp41. The products of the digestion were separated by gel electrophoresis to confirm insertion of the gp41/gp120 fragment into pcDNA6/V5-His B. This is shown by the fragment at approximately the correct molecular weight on the gel, as indicated in relation to the molecular weight markers on **Figure 5.1**. On confirmation, the plasmid could then be used for site-directed mutagenesis.

Figure 5.1: Gel electrophoresis of digested pcDNA6/V5-HisB_gp41



Digestion of pcDNA6/V5 His B_gp41 constructs with the restriction enzymes was undertaken to ensure efficient ligation of the gp41 insert into pcDNA6/V5 His B. Gel electrophoresis demonstrates the products from the restriction digestion of pcDNA6/V5 His B_gp41 construct (1-4) and pcDNA6/V5 His B control (5). The unmodified pcDNA6/V5 His constituted the negative control. The gp41 bands appear at approximately 1200bp.

5.2.2 Site-directed mutagenesis reactions

Mutations associated with resistance to C34 and those which occurred following *in vitro* passage with C34-PEG₄-Chol were selected to understand their effects on virus susceptibility to C34-PEG₄-Chol. Mutagenic oligonucleotide primers were designed in reference to the NL4-3 reference sequence (**Figure 5.2**). Forward primers were designed as the complementary sequence to gp41, while reverse primers were designed as the reverse complement sequences and nucleotide codon substitutions were located centrally within the primer (**Table 5.1**). The primers were then used for site-directed mutagenesis reactions to create daughter plasmids of pcDNA6/V5-HisB_gp41 with mutated gp41, as described in section 2.10.

To confirm successful mutagenesis of the gp41 insert of pcDNA6/V5-HisB, the plasmids were sequenced using the primers, BGH-reverse and T7P-forward, confirming the presence of mutations. Mutations L33S, V38E and A71T are shown in **Figure 5.3** against the gp41 NL4-3 reference sequence.

Attempted site-directed mutagenesis to introduce the E136G and E148K mutations failed. On reviewing the location of these mutations, it was noted that their loci lay upstream to the gp41/gp120 fragment (7250 to 8465) selected by *NheI* and *BamHI*. Hence, the reaction could not be completed with the chosen gp41/gp120 fragment.

Figure 5.2: Full-length sequence of gp41 HIV *env* from the NL4-3 laboratory clone

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GCA GTG GGA ATA GGA GCT TTG TTC CTT GGG TTC TTG GGA GCA GCA GGA AGC ACT ATG GGC GCA GCG TCA ATG ACG CTG
ACG GTA CAG GCC AGA CAA TTA TTG TCT GAT ATA GTG CAG CAG CAG AAC AAT TTG CTG AGG GCT ATT GAG GCG CAA CAG
CAT CTG TTG CAA CTC ACA GTC TGG GGC ATC AAA CAG CTC CAG GCA AGA ATC CTG GCT GTG GAA AGA TAC CTA AAG GAT
CAA CAG CTC CTG GGG ATT TGG GGT TGC TCT GGA AAA CTC ATT TGC ACC ACT GCT GTG CCT TGG AAT GCT AGT TGG AGT
AAT AAA TCT CTG GAA CAG ATT TGG AAT AAC ATG ACC TGG ATG GAG TGG GAC AGA GAA ATT AAC AAT TAC ACA AGC TTA
ATA CAC TCC TTA ATT GAA GAA TCG CAA AAC CAG CAA GAA AAG AAT GAA CAA GAA TTA TTG GAA TTA GAT AAA TGG GCA
AGT TTG TGG AAT TGG TTT AAC ATA ACA AAT TGG CTG TGG TAT ATA AAA TTA TTC ATA ATG ATA GTA GGA GGC TTG GTA
GGT TTA AGA ATA GTT TTT GCT GTA CTT TCT ATA GTG AAT AGA GTT AGG CAG GGA TAT TCA CCA TTA TCG TTT CAG ACC
CAC CTC CCA ATC CCG AGG GGA CCC GAC AGG CCC GAA GGA ATA GAA GAA GAA GGT GGA GAG AGA GAC AGA GAC AGA TCC
ATT CGA TTA GTG AAC GGA TCC TTA GCA CTT ATC TGG GAC GAT CTG CGG AGC CTG TGC CTC TTC AGC TAC CAC CGC TTG
AGA GAC TTA CTC TTG ATT GTA ACG AGG ATT GTG GAA CTT CTG GGA CGC AGG GGG TGG GAA GCC CTC AAA TAT TGG TGG
AAT CTC CTA CAG TAT TGG AGT CAG GAA CTA AAG AAT AGT GCT GTT AAC TTG CTC AAT GCC ACA GCC ATA GCA GTA GCT
GAG GGG ACA GAT AGG GTT ATA GAA GTA TTA CAA GCA GCT TAT AGA GCT ATT CGC CAC ATA CCT AGA AGA ATA AGA CAG
GGC TTG GAA AGG ATT TTG CTA TAA

```

The gp41 (NL4-3) reference sequence is shown. Codons of amino acids selected for substitution by mutagenic oligonucleotide primers are highlighted in red. Reading from left to right, these are lysine position 33, valine position 38, alanine position 71, glutamic acid position 136 and glutamic acid position 148.

Table 5.1: Mutagenic oligonucleotide primers

Mutation	Amino acid	Substitution	Direction	Mutagenic primer	Temp	% GC conte	Pri Dir
L33S	L = TTA	S = AGT, AGC	Forward	G CTG ACG GTA CAG GCC AGA CAA AGT TTG TCT GGT ATA GTG CAG C	81.6	52	No
			Reverse	G CAG CAC TAT TCC AGA CAA ACT TTG TCT GGC CTG TAC CGT CAG C	81.6	52	No
V38E	V = GTG	E = GAA, GAG	Forward	GCC AGA CAA TTA TTG TCT GGT ATA GAG CAG CAG CAG AAC AAT TTG CTG AGG	81.3	45	No
			Reverse	CCT CAG CAA ATT GTT CTG CTG CTG CTC TAT ACC AGA CAA TAA TTG TCT GGC	81.3	45	No
A71T	A = GCT	T = ACT, ACC ACA	Forward	CTC CAG GCA AGA ATC CTG ACT GTG GAA AGA TAC CTA AAG G	78.1	48	No
			Reverse	C CTT TAG GTA TCT TTC CAC AGT CAG GAT TCT TGC CTG GAG	78.1	48	No
E136G	E = GAA	G = GGA	Forward	CA AGC TTA ATA CAC TCC TTA ATT GGA GAA TCG CAA AAC CAG CAA G	79.1	40	No
			Reverse	C TTG CTG GTT TTG CGA TTC TCC AAT TAA GGA GTG TAT TAA GCT TG	79.1	40	No
E148K	E = GAA	K = AAA	Forward	CG CAA AAC CAG CAA GAA AAG AAT GAA CAA AAA TTA TTG GAA TTA GAT AAA TGG GC	81.2	32.7	No
			Reverse	GC CCA TTT ATC TAA TTC CAA TAA TTT TTG TTC ATT CTT TTC TTG CTG GTT TTG CG	81.2	32.7	No

Mutagenic oligonucleotide primers were designed with reference to the NL4-3 sequence (**Figure 5.2**). Forward primers represent the complementary sequence of g with the desired mutation introduced. Reverse primers represent the reverse complement to the sequence with the desired mutation introduced centrally.

Figure 5.3: Sequence analysis of gp41 following site-directed mutagenesis to introduce mutations L33S, V38E and A71T

	10	20	30	40	50	60	70	80	..
NL4-3gp41	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	AVERYLKDQQ	LL
gp41_L33S	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQSLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	AVERYLKDQQ	LL
gp41_V38E	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIEQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	AVERYLKDQQ	LL
gp41_A71T	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	TVERYLKDQQ	LL

	90	100	110	120	130	140	150	160	..
NL4-3 gp41	GIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF
gp41_L33S	GIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF
gp41_V38E	GIWGCSGK	LICTTAVPWN	ASWSNKSLLX	IX-----	-----	-----	-----	-----	--
gp41_A71T	GIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN	WF

Sequencing of gp41 HR-1 sequences integrated into pcDNA6/V5 His B plasmid. The upper sequence is the gp41 reference (NL4-3 gp41). The second, third and fourth sequences show gp41 sequences integrated into pcDNA6/V5 His B which have undergone site-directed mutagenesis to introduce the L33S, V38E and A71T mutations respectively (highlighted in red). Sequence analysis beyond position 112 failed within the fragment modified with introduction of V38E. Sequencing was performed using BGH-reverse and TP7-forward primers. Site-directed mutagenesis reactions for E136G and E148K mutations failed.

5.2.3 Generation of NL4-3 with point mutations

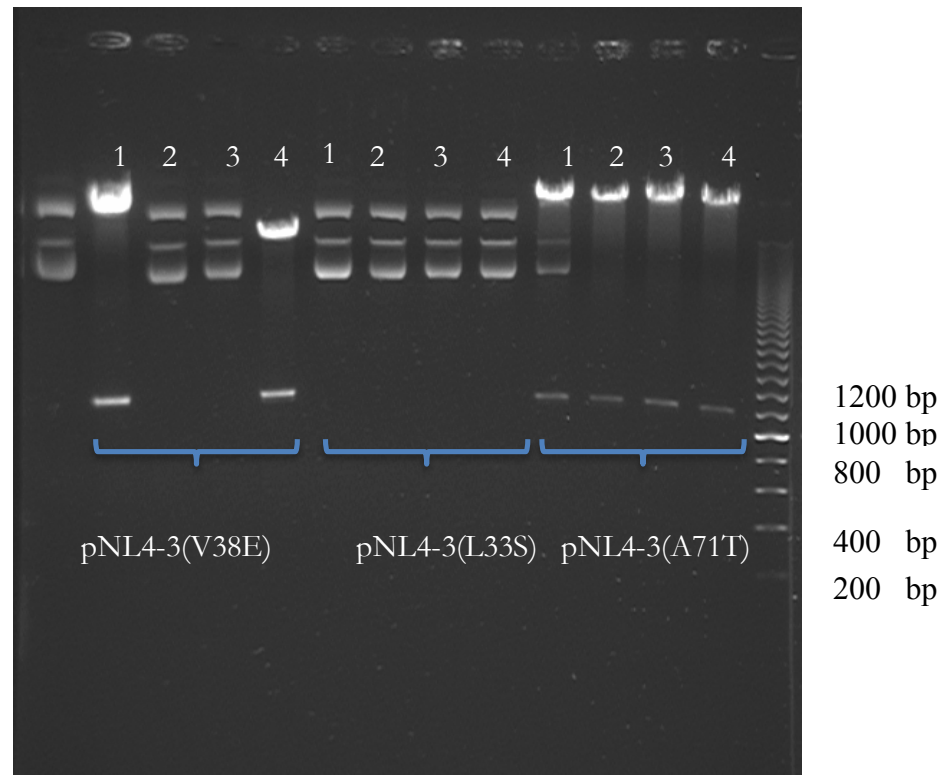
The mutated gp41/gp120 inserts were digested from the pcDNA6/V5-HisB plasmid using *NheI* and *BamHI* and separated by agarose gel electrophoresis. Simultaneously, un-mutated pNL4-3 plasmids were linearised using the same endonuclease restriction enzymes to remove the corresponding gp41/gp120 fragment. Following electrophoresis, gel bands were identified by their molecular weight under ultraviolet luminescence. The bands containing mutated gp41 and the pNL4-3Δgp41 were excised and purified, as described in section 2.1.9.3.

Sub-cloning of the mutated gp41/gp120 into pNL4-3Δgp41 was conducted with DNA ligase as described in section 2.1.11. After attempted ligation reactions between pNL4-3Δgp41 and gp41(L33S), gp41(V38E) and gp41(A71T), successful ligation was confirmed via a repeat small-scale digest with further gel electrophoresis; initial digestion suggested successful integration of gp41(A71T) in all four reactions and gp41(V38E) in two reactions (**Figures 5.4 and 5.5**).

For unsuccessful ligations, dephosphorylation of the 5' and 3' ends of the pNL4-3 with calf intestinal phosphatase (CIP) was used to prevent re-ligation of linearised plasmid DNA. Despite repeated attempts with CIP, however, gp41(L33S) could not be successfully ligated with pNL4-3Δgp41.

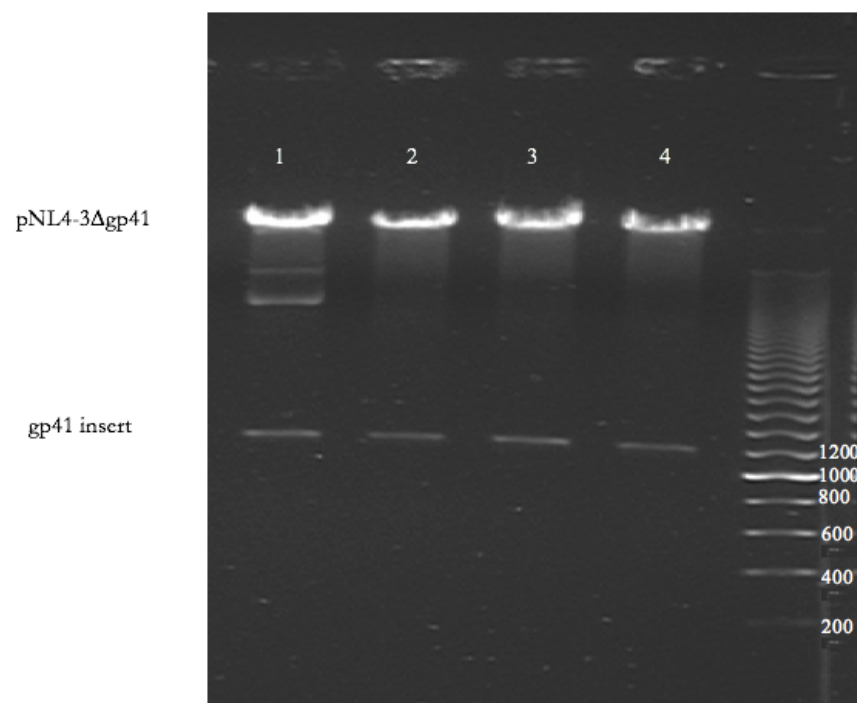
Where gel electrophoresis did not demonstrate the appearance of gp41 at the correct molecular weight under ultraviolet luminescence, sequencing was not attempted. The plasmids, pNL4-3(A71T) 1-4 and pNL4-3(V38E) 1 and 4 (**Figure 5.4**) were identified as potentially successful ligations and were sequenced to confirm ligation of the mutated gp41 with pNL4-3Δgp41 backbone.

Figure 5.4: Gel electrophoresis of the products of a small-scale digest to confirm ligation of pNL4-3 Δ gp41 with mutated gp41



Following the ligation reaction between the mutated gp41 insert with pNL4-3 Δ gp41, a small-scale digestion was undertaken of the ligation reaction products to verify successful integration. The figure shows an agarose gel of the electrophoresis of the small-scale digestion products. After illumination over ultraviolet light, gel electrophoresis suggested successful ligation of mutated gp41(A71T) 1-4 and gp41(V38E) 1 and 4 into the pNL4-3 Δ gp41 as illustrated by separation of the bands at the molecular weight 1200 bp, corresponding to the approximate molecular weight of the mutated gp41 insert. For pNL4-3(L33S) plasmids, failed electrophoresis likely represents incomplete digestion of plasmids.

Figure 5.5: Gel electrophoresis of the products of a small-scale digest to confirm ligation of pNL4-3Δgp41 with mutated gp41(A71T)



The recombinant pNL4-3 plasmid containing the gp41(A71T) insert was re-digested using *NheI* and *Bam*HI to demonstrate successful ligation; products were separated by electrophoresis along agarose gel to demonstrating the integration of the gp41 sequence into the linearised pNL4-3Δgp41.

5.2.4 Sequencing of mutated plasmids

Sequencing of the recombinant plasmids was undertaken using oligonucleotide primers developed for sequencing virus, as described in section 2.1.11.6. Full-length sequencing of pNL4-3(A71T) 1-4 demonstrated that only plasmids pNL4-3(A71T)2, pNL4-3(A71T)3 and pNL4-3(A71T)4 contained the A71T mutation (**Figure 5.6**). Sequencing of pNL4.3(V38E) failed to demonstrate successful integration of the gp41(V38E) and instead, showed unmodified pNL4-3.

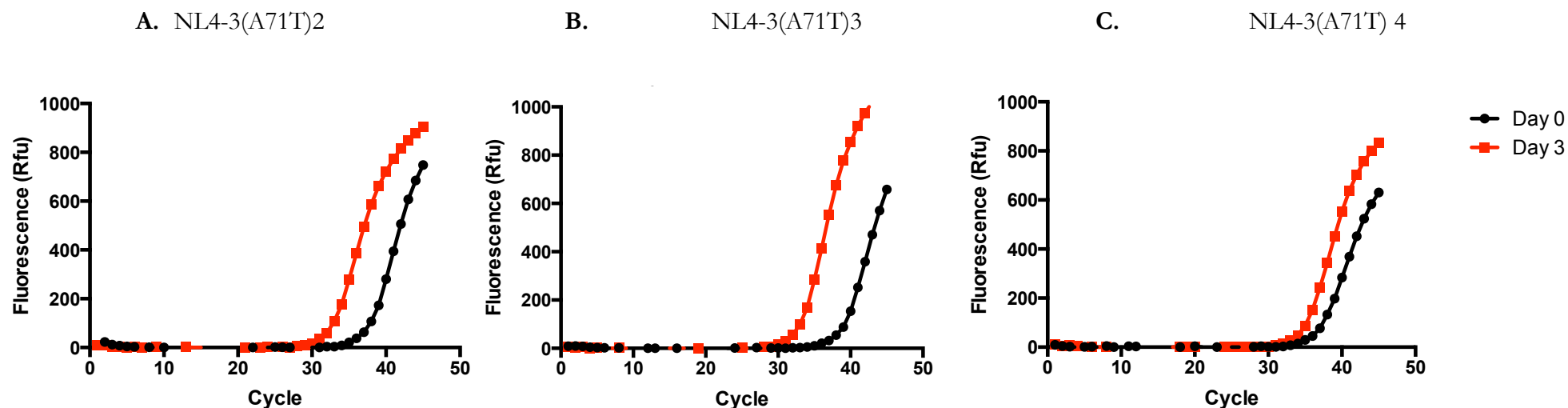
Virus stocks of NL4-3(A71T) were generated by transfection of the plasmids into TZM-bl cells, as described in section 2.1.11.3. Confirmation that the recombinant virus stock was replication-competent was carried out by culturing the virus in PM-1 cells over a period of 72-hours. This demonstrated increases in RT activity between day 0 and day 3 (as measured by the SG-PERT assay) (**Figure 5.7**). In addition, CPE began to develop in all cultures with PM-1 cells, thereby indicating successful viral culture.

Figure 5.6: Sequencing of recombinant pNL4-3 plasmids

								
	10	20	30	40	50	60	70	80
NL4_3_ref	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	AVERYLKDQQ
pNL4_3.1	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	AVERYLKDQQ
pNL4_3.2	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	TVERYLKDQQ
pNL4_3.3	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	TVERYLKDQQ
pNL4_3.4	AVGIGALFLG	FLGAAGSTMG	AASMTLTVQA	RQLLSDIVQQ	QNNLLRAIEA	QQHLLQLTVW	GIKQLQARIL	TVERYLKDQQ
								
	90	100	110	120	130	140	150	160
NL4_3_ref	LLGIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	ILSLIEESQN	QQEKNEQELL	ELDKWASLWN
pNL4_3.1	LLGIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN
pNL4_3.2	LLGIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN
pNL4_3.3	LLGIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN
pNL4_3.4	LLGIWGCSGK	LICTTAVPWN	ASWSNKSLEQ	IWNNMTWMEW	DREINNYTSL	IHSLIEESQN	QQEKNEQELL	ELDKWASLWN
								
	170	180	190	200	210	220	230	240
NL4_3_ref	WFNITNWLWY	IKLFIMIVGG	LVGLRIVFAV	LSIVNRVRQG	YSPLSFQTXL	PIPRGPDPRPE	GIEEEGGERD	RDRSIRLVNG
pNL4_3.1	WFNITNWLWY	IKLFIMIVGG	LVGLRIVFAV	LSIVNRVRQG	YSPLSFQTHL	PIPRGPDPRPE	GIEEEGGERD	RDRSIRLVNG
pNL4_3.2	WFNITNWLWY	IKLFIMIVGG	LVGLRIVFAV	LSIVNRVRQG	YSPLSFQTHL	PIPRGPDPRPE	GIEEEGGERD	RDRSIRLVNG
pNL4_3.3	WFNITNWLWY	IKLFIMIVGG	LVGLRIVFAV	LSIVNRVRQG	YSPLSFQTHL	PIPRGPDPRPE	GIEEEGGERD	RDRSIRLVNG
pNL4_3.4	WFNITNWLWY	IKLFIMIVGG	LVGLRIVFAV	LSIVNRVRQG	YSPLSFQTHL	PIPRGPDPRPE	GIEEEGGERD	RDRSIRLVNG

Sequencing of the recombinant plasmids, pNL4-3(A71T)1, pNL4-3(A71T)2, pNL4-3(A71T)3 and pNL4-3(A71T)4 aligned against the NL4-3 reference sequence (ref). Full-length sequencing of the plasmids are demonstrated extending across the FP, HR-1, HR-2 and extending to the transmembrane domain. Comparison with the reference sequence demonstrates successful integration of the A71T mutation into plasmids pNL4-3(A71T)2, pNL4-3(A71T)3 and pNL4-3(A71T)4.

Figure 5.7: Changes in RT activity of NL4-3(A71T) clones when cultured over 3 days



RT activity from three recombinant virus clones of NL4-3(A71T) grown in PM-1 cells was monitored by SG-PERT for 3 days. Increasing RT activity between day 0 and day 3 indicates the presence of replication-competent virus post-transfection of the plasmids, pNL4-3(A71T)2, pNL4-3(A71T)3 and pNL4-3(A71T)4 into TZM-bl cells.

5.2.5 Phenotypic effects of the A71T mutation

Undiluted and serial ten-fold dilutions (10^{-1} to 10^{-3}) of wild-type virus, NL4-3 and the NL4-3(A71T) recombinant virus were prepared and incubated in triplicate in TZM-bl cells in the presence of C34-PEG₄-Chol ranging from 0 nM to 3.2 nM. After 72-hours, the supernatants were removed, luciferase assay buffer was added and cultures were placed in -80 C freezer until frozen. The cultures were then thawed and read on a luminometer, as described in section 2.1.12. Luminescence results were read on an arbitrary scale (**Table 5.2 and 5.3**). Of note, viral entry into TZM-bl cells was the same when no drug was present when comparing NL4-3 with NL4-3(A71T) indicating equivalent fitness between wild-type and mutant-type virus.

Evidence of resistance conferred by the A71T mutation was most marked with undiluted virus, where inhibition of viral entry into TZM-bl cells appeared to occur from 0.1nM with wild-type NL4-3. With NL4-3(A71T) viral entry was only inhibited at drug concentrations above 1nM.

Based on luminescence results with undiluted virus, the estimated IC₅₀ of C34-PEG₄-Chol for NL4-3 was 0.2nM, compared with an IC₅₀ of 1.6nM for NL4-3(A71T); therefore, an approximate 8-fold resistance to C34-PEG₄-Chol is conferred by the A71T mutation. Statistically significant differences in susceptibility to C34-PEG₄-Chol were evident at the 10^{-1} and 10^{-2} dilutions. At 10^{-3} dilutions, luminescence signals were too weak to draw any conclusions (**Figure 5.8**).

Although the effects of the compensatory mutations, E136G and E148K, could not be studied, results from serial viral passages in the presence of C34-PEG₄-Chol, showed that with these mutations occurring alongside A71T, continuous viral growth was observed up to concentrations of 12.8nM with BaL (**Figure 4.4**). This suggests that their compensatory effects on restoring viral fusion kinetics and thus causing reduced susceptibility to C34-PEG₄-Chol are likely to be highly significant.

Table 5.2: Entry of NL4-3 and NL4-3(A71T) into TZM-bl cells at different concentrations of C34-PEG₄-Chol

Plate 1
NL4-3

C34-PEG₄-Chol concentration

Dilution	0nM	0nM	0nM	0.05nM	0.05nM	0.05nM	0.1nM	0.1nM	0.1nM	0.2nM	0.2nM	0.2nM
10 ⁰	400019	400019	400019	400019	400019	400019	400019	400019	400019	253318	231354	223459
10 ⁻¹	373915	400019	395929	106391	112438	125558	73956	47278	30718	27152	37419	23745
10 ⁻²	45706	42879	35146	13309	20823	15053	13336	10034	9296	9906	8756	9927
10 ⁻³	10281	18931	8895	9148	15112	9471	7826	9348	9611	9608	9898	10066

NL4-3(A71T)

C34-PEG₄-Chol concentration

Dilution	0nM	0nM	0nM	0.05nM	0.05nM	0.05nM	0.1nM	0.1nM	0.1nM	0.2nM	0.2nM	0.2nM
10 ⁰	400019	400019	400019	400019	400019	400019	400019	400019	400019	400019	400019	400019
10 ⁻¹	400019	400019	400019	326985	380387	362569	258676	114305	167475	166170	166724	102871
10 ⁻²	60960	68797	53563	31763	27725	25275	21788	25376	26233	21360	21081	15570
10 ⁻³	10742	7387	18108	7413	6650	6918	7031	7127	10107	6744	6836	10600

Entry of NL4-3 and NL4-3(A71T) into TZM-bl in different concentrations of C34-PEG₄-Chol was measured by recording luminescence emitted from cells after application of luciferase buffer. Measurements were recorded in triplicate at each concentration of drug. Four serial 10-fold dilutions (10⁰ to 10⁻³) of virus were tested.

Table 5.3: Entry of NL4-3 and NL4-3(A71T) into TZM-bl cells at different concentrations of C34-PEG₄-Chol

Plate 2

NL4-3

C34-PEG₄-Chol concentration

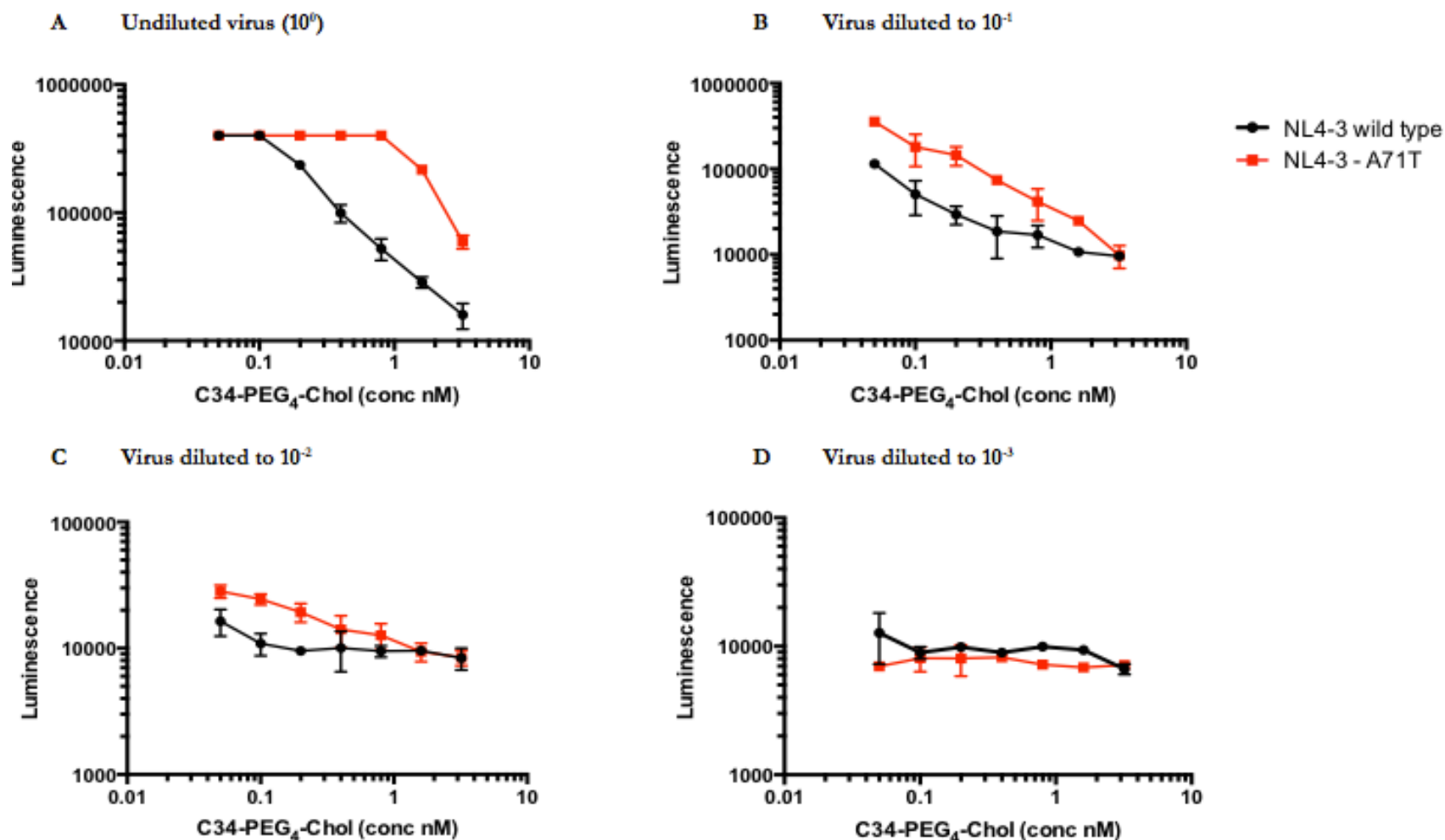
	0.4nM	0.4nM	0.4nM	0.8nM	0.8nM	0.8nM	1.6nM	1.6nM	1.6nM	3.2nM	3.2nM	3.2nM
10 ⁰	107421	81375	109697	64034	47113	46174	28142	26108	31623	13005	14840	19963
10 ⁻¹	11321	29602	15038	17623	11786	21410	9723	11328	11208	10583	9140	9061
10 ⁻²	7704	14136	8334	10433	9553	8471	10269	9544	8915	9626	8953	6518
10 ⁻³	8952	8879	8997	9411	10920	9502	9743	9657	8644	7084	5936	6952

NL4-3.A71T

C34-PEG₄-Chol concentration

	0.4nM	0.4nM	0.4nM	0.8nM	0.8nM	0.8nM	1.6nM	1.6nM	1.6nM	3.2nM	3.2nM	3.2nM
10 ⁰	400019	400019	400019	399911	399934	398807	220203	214199	215503	51093	62364	64572
10 ⁻¹	77659	69427	74854	59827	38241	26858	23444	23868	26850	13157	8105	8135
10 ⁻²	18429	13322	10494	9569	12852	15498	7600	10282	10296	8615	9545	7212
10 ⁻³	8534	8193	7936	7198	6800	7616	6654	7197	6720	6806	7846	6785

Figure 5.8: Inhibition of NL4-3 and NL4-3(A71T) entry into TZM-bl cells by C34-PEG₄-Chol measured by luminescence



Inhibition of entry of wild-type NL4-3 and NL4-3(A71T) into TZM-bl cells in the absence of drug up to concentrations of 3.2 nM of C34-PEG₄-Chol. Compared with NL4-3, reduced susceptibility of NL4-3(A71T) to C34-PEG₄-Chol is clearly demonstrated with undiluted virus (A) and serial dilutions of 10^{-1} (B) and 10^{-2} (C).

5.3 Discussion

The above results prove, at least partially, the hypothesis that mutations occurring in the presence of C34-PEG₄-Chol confer reduced susceptibility of HIV to the drug. Phenotypic analysis suggests that the A71T substitution is implicated in resistance to C34-PEG₄-Chol. Although the interface between C34-PEG₄-Chol and the PD lies between residues 33 and 66, the C34 lead molecule incorporates the PBD and traverses the entire PD up to residue 70. Thus, the A71T substitution occurs immediately adjacent to the highly conserved PD. It is likely, therefore, that such a substitution lying adjacent to this functional region will have an impact on the integrity of the PD and, therefore, its interaction with the PBD. It follows that this substitution may affect the fusion kinetics of the viral-cell fusion pathway.

It is acknowledged that gp41 fragment containing the A71T mutation was inserted into a pNL4-3Δgp41 backbone plasmid while the mutation occurred within the BaL laboratory clone; no mutations were noted to have occurred with passages of NL4-3. Although it would have been preferable to use a BaL plasmid to insert the gp41_A71T fragment into, this was not possible. By comparing NL4-4(A71T) with NL4-3 however, this offered a direct comparison of the phenotypic effects of this mutation as no other mutations were present which. Thus it has been assumed that a similar effect would have been noted had it been possible to create BaL(A71T) for comparison with BaL.

The failed ligation of the mutated gp41(L33S) and gp41(V38E) with pNL4-3Δgp41 could not be resolved within the time limits of this study. Repeated attempts were undertaken to digest, extract and purify the un-mutated gp41 from pNL4-3 before ligation with the gp41(L33S) and gp41(V38E) mutants. Furthermore, to prevent possible re-ligation of the ends of the linearised pNL4-3Δgp41 at phosphorylated 3' and 5' ends, CIP was added to dephosphorylate and prevent this but without success. Repeated site-directed mutagenesis to introduce L33S and V38E into the pcDNA6/V5-HisB_gp41 construct was not undertaken. Although, the reasons for these failed experiments were unclear, it is possible that the failed ligation of the gp41(L33S) and gp41(V38E) may have resulted from a problem intrinsic to the gp41 mutants within pcDNA6/V5-HisB_gp41 since this was the only step not repeated.

Results from studies by Armand-Ugon *et al*, 2003 with the native C34 molecule have demonstrated profound resistance to be induced by these two mutations [146]. In these experiments, the HXB2-

derived strain was rendered >1000-fold resistant to the native C34 molecule with the V38E substitution, while the NL4-3 derived strain containing the L33S substitution was >500-fold resistant. Nevertheless, it cannot be inferred what the effects of these mutations on C34-PEG₄-Chol would be due to the structural differences between C34 and C34-PEG₄-Chol. Secondly cholesterolisation of C34-PEG₄ could cause enhanced affinity of the molecule to lipid rafts where viral entry occurs. Notwithstanding, it seems likely that multiple substitutions across HR-1 domain may act to enhance viral fitness in the presence of C34 and, by inference, C34-PEG₄-Chol and that these substitutions may confer resistance by different mechanisms.

Unfortunately, phenotypic effects of the compensatory mutations could not be studied with the constructs developed. Site-directed mutagenesis failed to introduce E136G and E148K mutations owing to the gp41/gp120 fragment only being comprised of residues from positions 7250 to 8465 of pNL4-3. It seems likely, however that E136G and E148K contribute significantly to resistance to C34-PEG₄-Chol. This is supported by the fact that within viral passage experiments, there was continued viral growth until drug concentrations of around 12.8nM.

Similar observations have been made with previously documented double mutations such as the N43D/S138A where resistance has been markedly enhanced by the presence of a compensatory mutation [149].

More recently the occurrence of E136G substitution with L57R and E49K substitutions has been documented in response *in vitro* passage experiments with a novel short peptide fusion inhibitor, MTSC22 [148]. Together L57R/E136G conferred a 836-fold reduction in susceptibility to the peptide (compared with 402-fold reduced susceptibility by L57R alone). When E136G was introduced as a single mutation into NL4-3, moderate resistance to the peptide was also conferred with a 4.7-fold reduction in susceptibility. Contrary to what might be expected, when introduced as a single mutation, E136G decreased the helical stability of 6-HB structure on evaluation by circular dichroism [164]. In contrast, results from the present study with C34-PEG₄-Chol might suggest that both E136G and E148K when occurring with A71T might restore the conformational stability of the HR-1/HR-1 6HB. However, circular dichroism which would assess this is beyond the scope of this thesis.

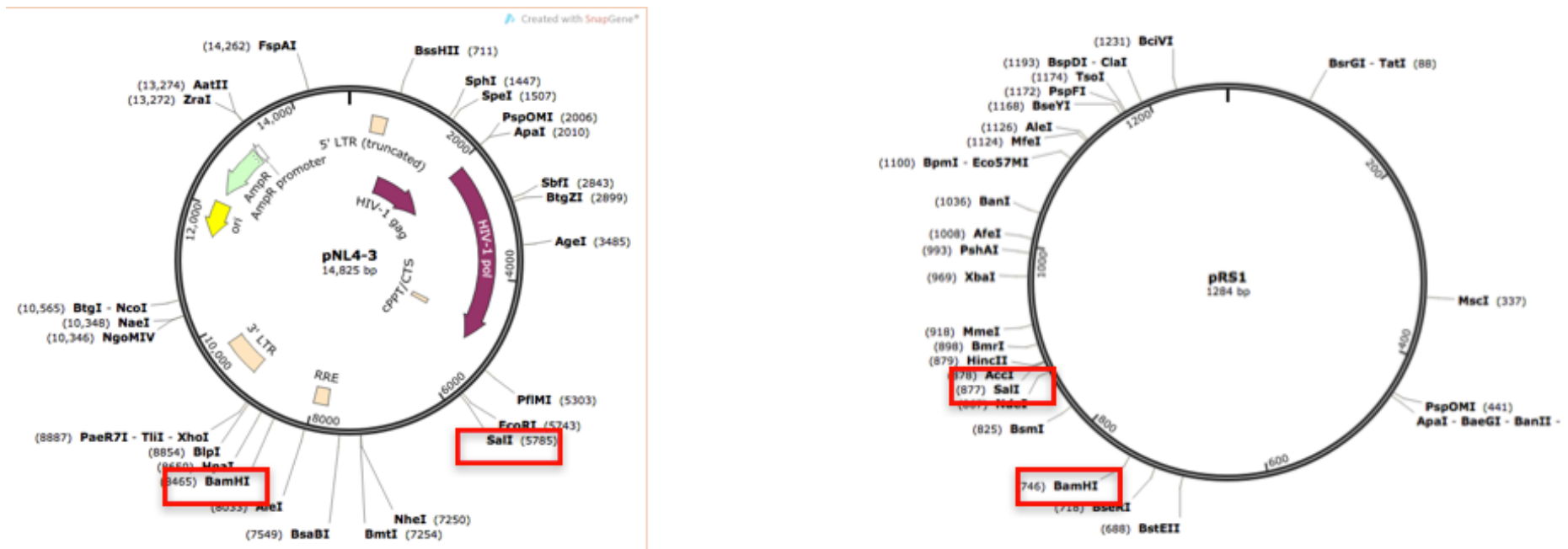
While site-directed mutagenesis protocols are most widely used for cloning, other strategies could have helped overcome the problems encountered. Similar studies investigating the effects of

mutations within *env* on susceptibility to entry inhibitors have utilised the alternative plasmid, pRS1 which allows construction of the analogous pRS1_gp41. This has allowed incorporation of the *Sa*II-*Bam*HI gp41/gp120 fragment of pNL4-3 extending from 5785 to 8854 [150]. By doing so, mutagenesis to effect the substitutions E136G and E148K at positions 8162-8165 and 8199-8201, respectively could have been achieved (**Figure 5.9**). The ability to do so however, was hampered by highly limited availability of this plasmid.

As an alternative approach, splicing by overlap extension PCR (SOEing PCR) allows recombination of DNA fragments without relying on endonuclease restriction site. In this technique, conventional PCR is used to generate double-stranded linear products. Two external mutagenic SOEing oligonucleotide primers modify the 5' termini of the two PCR products so that they have share homology. Under PCR conditions, the homologous ends of the two fragments can then hybridise. A recombinant product is formed when the fragments are extended by polymerase during thermal cycling. This full-length product can then undergo exponential amplification with two oligonucleotide primers by PCR and, with appropriate modification, ligated into the desired plasmid (**Figure 5.10**).

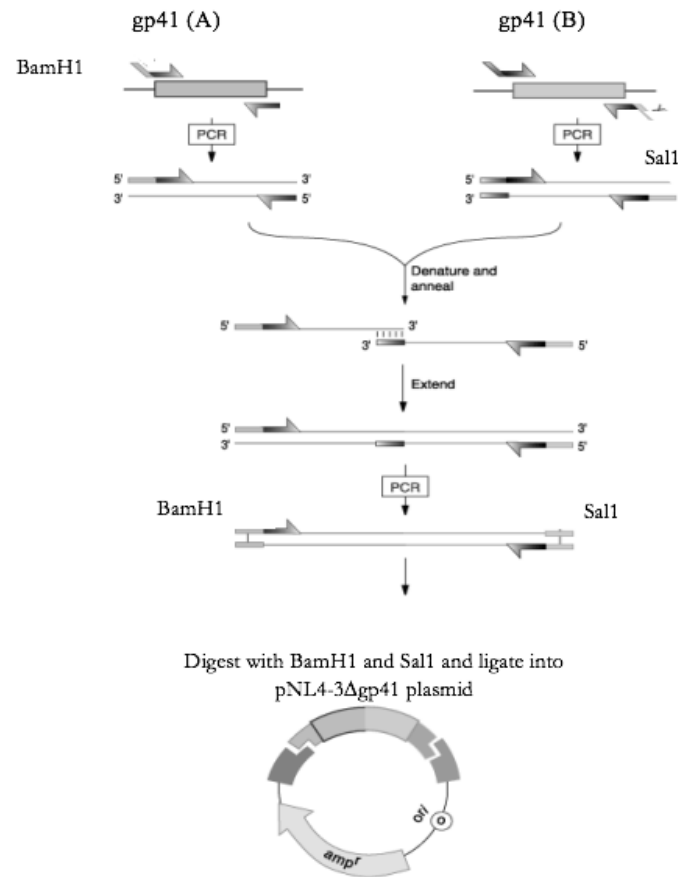
As further work is performed on the development of HR-2-based FIs in the future, the effects of the aforementioned resistance pathways merit further investigation.

Figure 5.9: Schematic of pNL4-4 and pRS1 plasmids



An alternative plasmid recombinant, pRS1_gp41 could be constructed by digestion of the BamH1/SalH1 fragment from pNL4-3 and ligating with pRS1 following extraction of its BamH1/Sal1 construct. This construct would have allowed site-directed mutagenesis to introduce the E136G and E148K mutations into gp41.

Figure 5.10: Diagram of mutagenesis by splicing by overlap extension (SOEing) technique



Product is PCR amplified from gp41(A) and from gp41 (B). SOEing primers are used to modify the ends of the two PCR products so that they are homologous. Products are then mixed, denatured and annealed: the top strand and bottom strands overlap and act as primers for one another. The recombinant product is formed when polymerase extends the overlap. The recombinant fragment is then digested with endonuclease restriction enzymes to allow ligation into pNL4-3Δ gp41.

Chapter 6

**The development of an adaptive design for
a Phase 1 trial of C34-PEG₄-Chol**

6.1 Introduction

Phase 1 clinical trials represent the first exposure of humans to a new drug. Their aim is to determine the drug's safety, tolerability and model its pharmacokinetic profile thereby establishing the optimal dose and dosing schedule for the drug. In some instances, there may be investigation of the pharmacodynamics of the drug. The design of a phase 1 trial has a number of components which include determining the starting dose, the increments between doses, dose escalation methods, determining the number of patients to receive each dose, establishing stopping rules and defining dose limiting toxicities (DLT). Because the underlying premise is that the higher the dose, the more efficacious a drug will be, the ultimate aim is to determine the MTD or minimum effective dose (MED) for use in subsequent phases of clinical trials of the drug.

Although exceptions exist, generally, phase 1 studies are conducted in healthy male subjects with no, or only minor, medical problems. In doing so, the pharmacokinetic profile is modelled in a uniform population and limits the variability introduced by differences in body habitus, volume of distribution, elimination and physiology altered by disease state. Moreover, it avoids exposing a new drug to women of potential child bearing age. Despite these advantages, such an approach limits the understanding of the pharmacokinetic profile of a drug when used in a population with the target disease and also in women until later phase trials. While avoiding exposing women of childbearing potential to new drugs is of paramount importance, it remains desirable to study new ARV drugs in the target population i.e. PLWHIV.

Phase 1 dose-escalation clinical trials are broadly defined as 1) traditional rule-based and 2) adaptive designs (the latter of which can also be subdivided into rule-based and model-based).

Rule-based methods apply pre-specified rules to observations of clinical data to assign participants to dose levels [165]. The clinical data on which decisions are based are usually the primary endpoint, for example MTD or MED. Typically, phase 1 rule-based dose-escalation studies begin with a dose considered safe based on pre-clinical data. No assumptions are made on the dose-toxicity relationship prior to initiating the study. Within oncology studies, this starting dose is typically one-tenth of the dose which is lethal to 10% of the most sensitive animal species in preclinical studies [166]. Using an oncology study as an example, a pre-specified number of patients (typically $n=3$) are administered the drug at the starting dose. Participants are observed for toxicity and if none are

observed, a dose increment occurs with the next cohort of patients. This process continues until DLTs occur. Classically, dose increments are calculated as multiples of the Fibonacci sequence such that, initially, large increments occur but become narrower as the study proceeds [165]. In the classic example of a cohort of three, if two or more participants experience a DLT, a dose reduction is performed so that another cohort of patients ($n=3$) are exposed to the initial dose. If no further toxicities are observed at this dose, the MTD is then chosen as the dose immediately below the level at which DLTs occur. The most widely cited example of such a design is the '3+3' design. Such designs, however, may have a long duration especially when the starting dose is likely to be far below the therapeutic level.

In an effort to expedite accrual of patients into trials, prevent exposing large numbers of patients to subtherapeutic doses and to characterise the pharmacokinetic profile of drugs at their therapeutic dose, various alternative rule-based designs have been proposed [165]. Examples include enrolling one patient at a time before incrementing the dose until one participant develops DLTs whereupon there is a reversion to the '3+3' designs. Indeed, the importance of these approaches is amplified when financial and logistical reasons mean that only a limited number of patients can be recruited to a trial. Consequently, to maximise the accrual of clinically useful pharmacokinetic data, it is imperative to expose the greatest number of participants to the MTD or MED in order to characterise the dose-toxicity curve as extensively as possible.

To date, phase 1 clinical trial design with new ARV drugs have typically followed a traditional rule-based approach and have not utilised the alternative trial designs seen within fields of oncology. In addition, by convention, most phase 1 trials of new ARV drugs have been conducted in HIV-negative individuals. By performing a phase 1 trial in the target HIV-positive cohort, the efficiency of obtaining pharmacokinetic and pharmacodynamic data is enhanced by overcoming the need to repeat a trial which has already been performed in a healthy population.

In 2011, a grant application was submitted to the Medical Research Council (MRC) for a two stage, phase 1, first-in-man clinical trial of C34-PEG₄-Chol. For the first stage, it was proposed to recruit 28 HIV-positive men to receive a single dose of C34-PEG₄-Chol across the dose range of 10-80 mg. A sample size of 28 subjects comprising of 8 placebo and 20 active subjects was calculated to provide 80% power to observe adverse events which have a population incidence of $\geq 30\%$.

Based on experience with enfuvirtide, T1249 and SFT [78, 113, 123, 167], DLTs were not expected with a single dose of C34-PEG₄-Chol within the proposed dose range. In addition, experience with enfuvirtide suggested that the occurrence of ISRs increased as the duration of use increased. Thus, it was felt unlikely that ISRs would prove to be a significant problem following a single subcutaneous dose. It was, therefore, proposed to examine the probability of dose-inefficacy, as opposed to dose-toxicity.

A traditional dose-escalation model was proposed with four doses, namely 10mg, 20mg, 40mg and 80mg. A standard '5+5+5+5' double-blind clinical trial design was suggested whereby, at each dose level, 5 participants would receive active drug (and 2 participants would receive a placebo). Within the '5+5+5+5' design, the first 5 participants would receive 10 mg dose. Pharmacokinetic analysis would be undertaken to determine if MED had been achieved. As previously described, the MED has been defined as lowest dose which achieves a plasma levels 10 x time above the IC₅₀ at D4 after administration. If any of the 5 participants failed to achieve the MED, a dose increment to the next level would be undertaken to 20 mg. Incremental dosing with the cohorts would continue until no failures were observed, and this dose would be chosen to take forward to stage 2. All dose escalations would additionally be subject to satisfactory safety data review. Within stage 2, the optimal dose would be trialed in a multi dose stage in which with four consecutive doses.

Based on pre-clinical data, however, it was anticipated that the lowest dose, 10 mg, would be subtherapeutic whereas 80 mg is the maximal deliverable dose of drug in solute. Consequently, should the '5+5+5+5' clinical trial design be followed, a significant number of patients were likely to be exposed to subtherapeutic doses thereby limiting the characterisation of the dose-response curve at the optimal dose. Moreover, the initial design only explored four possible dose levels and did not allow for selection of a dose between these levels.

Pre-specified within funding application, however, it was proposed to investigate alternative clinical trial designs to the traditional rule-based designs. In doing so, it was proposed to undertake simulations of approaches which may lead to improved characterisation of the pharmacokinetic profile of the optimal dose. Upon satisfactory review of the simulations from both a statistical and logistical perspective, it was suggested that a novel approach to the clinical trial may be adopted and that changes in the number of patients allocated to each dose group may occur.

Adaptive clinical trial designs rely on statistical estimation of the primary endpoint (for example, MTD or MED) to assign participants to a dose level. In contrast to rule-based clinical trial designs

where no assumptions are made on the dose-response curves, in adaptive model-based designs an assumed dose-response curve is generated *a priori* [168]. Within the model, as data is accrued from each participant, this is fed back into a statistical algorithm and the dose-response curve evolves in an iterative process to re-estimate the primary endpoint.

The continual reassessment method (CRM) is a type of adaptive design. The premise of a CRM is that the probability of toxicity and efficacy both increase as the dose increases. Like other adaptive designs, the dose-response curve is therefore hypothesised in advance and a target toxicity level (TTL) or target inefficacy level (TIL) is chosen. The initial dose-response curve is modified as data accrued from participants is fed back into the statistical algorithm [168]. The adaptive model should therefore aim to estimate the level at which DLTs occur, and choose the MTD as that immediately below this level. In the case of a dose-inefficacy curve, the MED is estimated according to the intercept between the curve and the required TIL (e.g. 5% TIL), and this dose or above should be chosen to bring into subsequent phases of trials (**Figure 7.1**).

The initial dose-response curve is modified as data accrued from participants is fed back into the statistical algorithm. From the data fed back into the adaptive model, it is aimed to dose the subsequent patient or cohort of patients at the level most likely to be closest to the DLT, or where inefficacy is measured, at or above the MED [168].

Since its initial description [169], the CRM has drawn criticism in that large dose increments could be chosen based on feedback through a statistical model with data from few patients. Consequently, modified CRMs have been developed which incorporate rules to prevent dose escalations which result in overshooting the DLT level [170-172]. Examples of such modifications to CRM include:

- choosing the lowest starting dose.
- not exposing a patient to a dose increment if the participant immediately before them has experienced a toxicity.
- dosing at least 2 participants at the same dose especially at higher doses.
- ‘escalation with overdose control’ (EWOC) where the MTD is continually re-estimated with accruing data, for example, after every 1 patient (or every 2 or 3 patients) and the new estimate of the MTD informs the decision on which dose to allocate to the next patient.

Based on experience with enfuvirtide, T1249 and SFT, DLTs were not expected with a single dose of C34-PEG₄-Chol within the range of 10-80 mg. In addition, experience with enfuvirtide

suggested that the occurrence of ISR increased as the duration of use increased. Thus, it was felt unlikely that ISRs would prove to be a significant problem following a single subcutaneous dose. While safety parameters would be continually assessed throughout, it was therefore proposed to modify the statistical approach such that a pharmacokinetically-guided endpoint would be used to drive dose selection within the statistical algorithm. Development of the modified CRM involved a collaboration between clinicians and statisticians. Statistical elements of the design, including the development of the modified CRM algorithms were conducted by Professor Deborah Ashby's group, led by Dr Alexina Mason. In developing the CRM, the clinical team were required to 1) describe the probable dose-inefficacy curve for C34-PEG₄-Chol *a priori* and 2) identify an acceptable TIL.

Hypothesis

Alternative clinical trial designs will yield more useful clinical data compared to a standard clinical trial design.

Aims

The aims of this chapter are therefore:

1. to describe the clinical input in the design of a adaptive design (modified CRM) for stage 1 of a phase 1 clinical trial of C34-PEG₄-Chol.
2. to compare the results of simulations of the rule-based and model-based adaptive designs with the traditional '5+5+5+5' design.
3. to evaluate the advantages and disadvantages in dose selection that the adaptive designs may hold over a '5+5+5+5' design based on statistical simulations performed.

6.2 Study design

The proposed structure of stage 1 and study endpoints are described in sections 3.7 and 3.7.1 respectively. A hybrid design between rule-based and adaptive design was proposed for stage 1. A full description of the methodology in design of the modified CRM is described in Mason A *et al*, 2016.

6.2.1 Substage 1

Prior to undertaking the adaptive CRM, a double-blind, rule-based ‘2+2+2+2’ dose-escalation and safety phase was agreed. For assessment of safety endpoints, 2 participants were to be dosed simultaneously where one participant received active drug and one participant received placebo. An observation interval of at least 4 days was planned between each cohort of two participants in order to assess for DLTs. Assuming no adverse reactions were noted, a further two participants would be dosed at the same level, and, if no adverse reactions occurred, a dose escalation would then be undertaken (**Table 6.1**). Before each dose escalation, a review by the data safety and monitoring board (DSMB) was planned. Within this rule-based design, it was aimed to assess safety across the entire dose range (10-80 mg).

Table 6.1 Stage 1, substage 1 single dose administration per dose group

Time interval between groups of participants	
Initial participant dosing	Participants 1 and 2*
Interval	At least 4 days later
Subsequent participant dosing	Participants 3 and 4 *
* one placebo will be included in each of these groups	
Dose safety analysis prior to dose escalation	

6.2.2 Substage 2

A modified CRM was planned for substage 2.

In this dose finding study, the aim was to determine the MED, with the assumption that the higher doses are more effective than lower doses, and all doses above MED are effective. As all the placebo doses had been allocated in substage 1, only active drug would be administered in substage 2. Due to the need for less information to precisely model the area near the MED, a one-parameter

logistic curve was chosen *a priori* as the functional form of the dose-inefficacy curve (**Figure 6.1**).

6.2.2.1 Dose levels

It was proposed that eight dose levels across the dose range 10-80 mg could be assigned to participants (i.e. 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg or 80 mg).

6.2.2.2 Cohort sizes

Cohorts of 2 participants would be dosed simultaneously.

6.2.2.3 Identification of the target dose

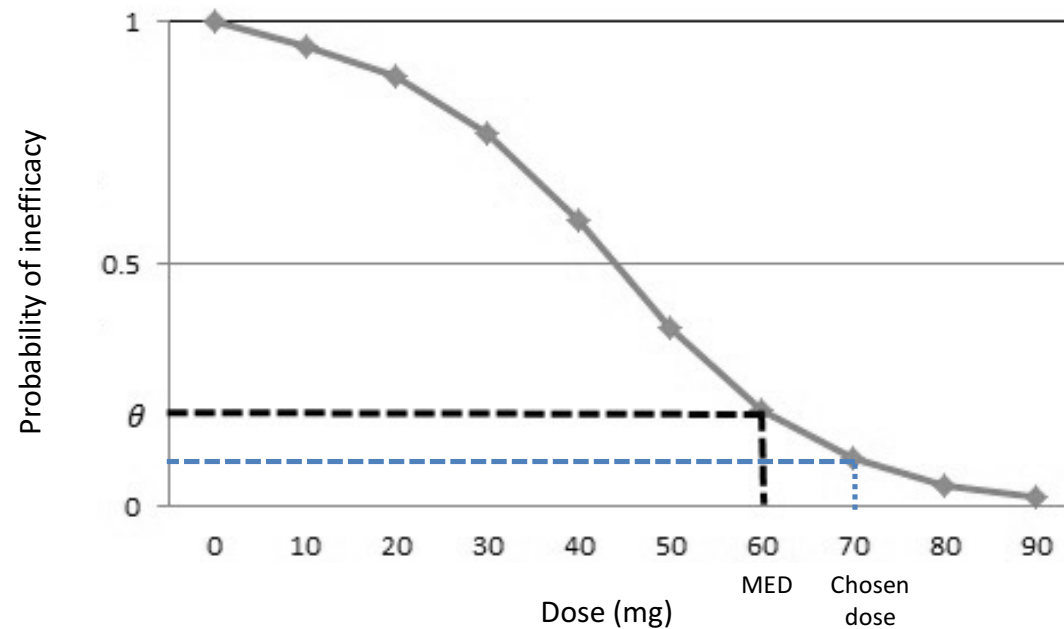
After modelling the dose-inefficacy curve, the MED is estimated by defining the acceptable TIL and selecting that dose (or higher) at the intercept between the TIL for use in stage 2. From a clinical perspective, it was considered that the TIL should be no higher than 5%. The statisticians implemented two approaches to selecting the dose to take forward after modelling the dose-inefficacy curve:

- Rule-based where the lowest dose is selected which does not allow any failures in achieving the endpoint at this dose or any higher doses (the choice is restricted to 10 mg, 20 mg, 40 mg and 80 mg and any other doses used in the adaptive design).
- Model-based where the lowest dose is selected with a probability of inefficacy *below* the TIL (dose levels to be simulated include 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg and 80 mg).

6.2.3 Substage 3

Two participants were kept as reserves in case insufficient data was gathered to model the pharmacokinetic profile of the MED chosen by the adaptive design after the first 18 participants had received active drug. These participants would be dosed at the dose chosen for stage 2 if deemed necessary to gain more data at the end of substage 2.

Figure 6.1 Predicted (*a priori*) dose-inefficacy curve



The figure shows the *a priori* dose-inefficacy curve. The curve is modified as D4 pharmacokinetic data is fed back into the statistical model to provide an estimate of the MED. Here, θ represents the TIL and it is achieved at the 60 mg. According to the model-based decision for dose identification, however, 70 mg would be the dose chosen to take forward as this is the lowest dose with the probability of inefficacy *below* the TIL.

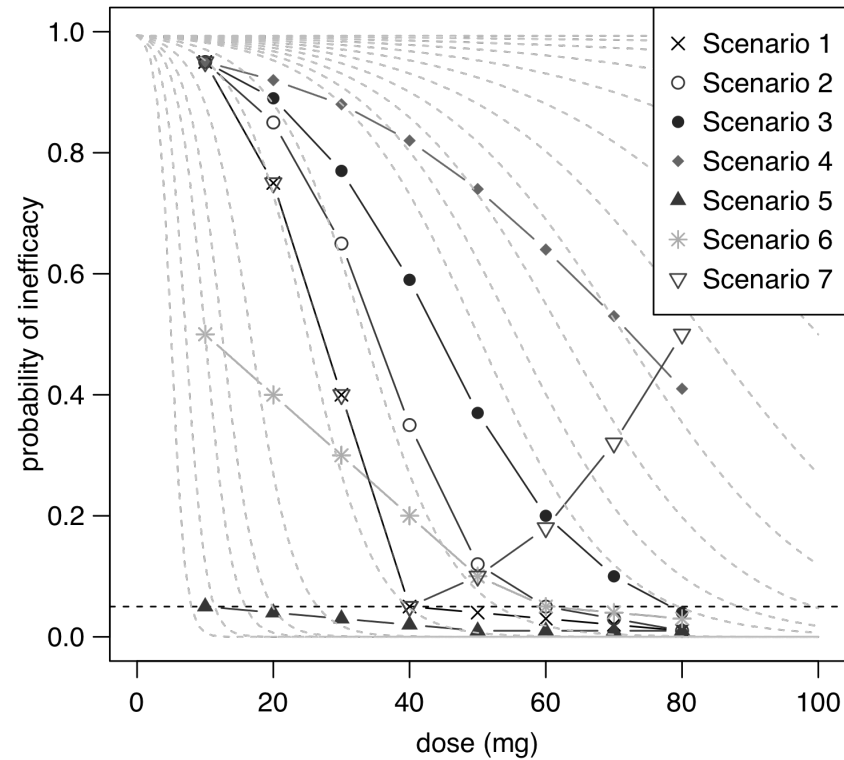
6.3 Test scenarios

Seven scenarios were devised to assess the performance of the adaptive design in selecting the MED at various TILs through simulations. The scenarios are depicted in **Figure 6.2** and were as follows:

1. Optimal dose in the middle of the dose range and selected as one of the doses to be trialled in substage 1 (MED at 5% TIL = 40 mg).
2. Optimal dose higher than scenario 1 and not selected as one of the doses to be trialled in substage 1 (MED at 5% TIL = 60 mg).
3. Optimal dose at the upper boundary of the dose range (MED at 5% TIL = 80 mg).
4. No dose is effective (MED at 5% TIL is not applicable).
5. All doses effective (MED at 5% TIL = 10 mg).
6. Shallower dose inefficacy curve than observed with other scenarios (MED at 5% TIL = 60 mg).
7. MED at 5% TIL (40 mg) is effective, higher doses ineffective (e.g. through auto-induction of metabolism of drug).

The TILs for investigation were 5%, 10% and 20%.

Figure 6.2 Test scenarios



The figure shows seven possible scenarios to test the efficiency of the adaptive design in identifying the MED. The dashed grey lines show members of the family of one parameter logistic curves specified for the proposed adaptive models. The horizontal dashed black line indicates the 5% TIL; the MEDs lie on the 5% TIL line (*figure reproduced courtesy of Dr Alexina Mason, LSHTM*).

6.4 Results

Simulations were performed to compare the performance of the adaptive design (at three TILs, 5%, 10% and 20%) with the '5+5+5+5' design across the seven scenarios. For each scenario, 1,000 realisations were performed with the adaptive and '5+5+5+5' designs. A full description of simulations is provided in Mason A *et al*, 2017 [173].

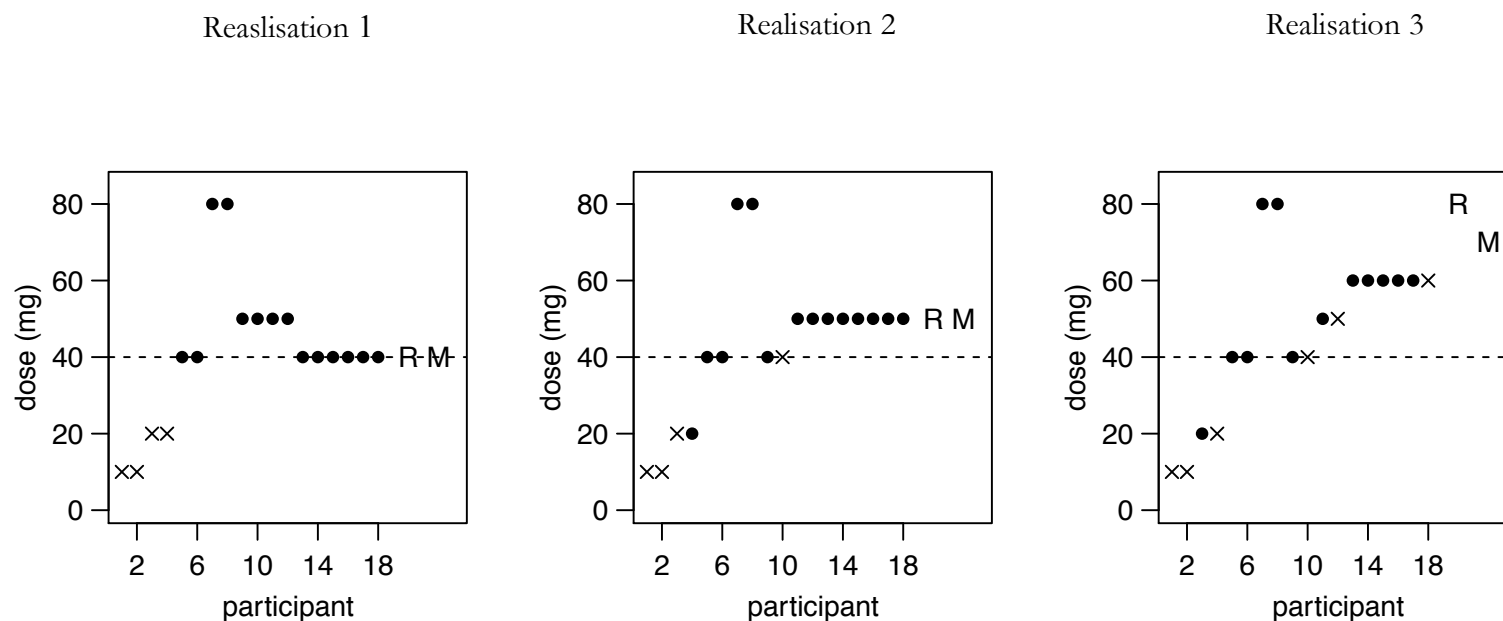
Within realisations, cohorts of 2 participants received C34-PEG₄-Chol at one of the doses under consideration. The aim was to challenge the adaptive design to estimate the correct MED in each realisation according to the simulated D4 pharmacokinetic results. The design which most reliably estimated the MED across all the realisations in the seven scenarios would then be incorporated into substage 2 of the clinical trial.

Figure 6.3 is an illustration of the output from the adaptive design (at a 5% TIL) for three simulations of scenario 1.

For scenario 1, the MED lies at a dose of 40 mg. Within substage 1, eight participants receive active drug at the levels of 10 mg (n=2), 20 mg (n=2), 40 mg (n=2) and 80 mg (n=2), as depicted within the first three simulations (**Figure 6.3**). Based on the simulated D4 pharmacokinetic results from participants in substage 1, the adaptive design modifies the dose-inefficacy curve (**Figure 6.4**) and assigns subsequent doses to the MED estimated from the intercept of the 5% TIL with the dose-inefficacy curve, as previously demonstrated in **Figure 6.1**. Modification of the curve and re-estimation of the MED continues as new simulated D4 pharmacokinetic results are entered into the model. Consequently, the 90% confidence intervals (CIs) also become narrower as a greater degree of certainty emerges about which doses lie below the 5% TIL.

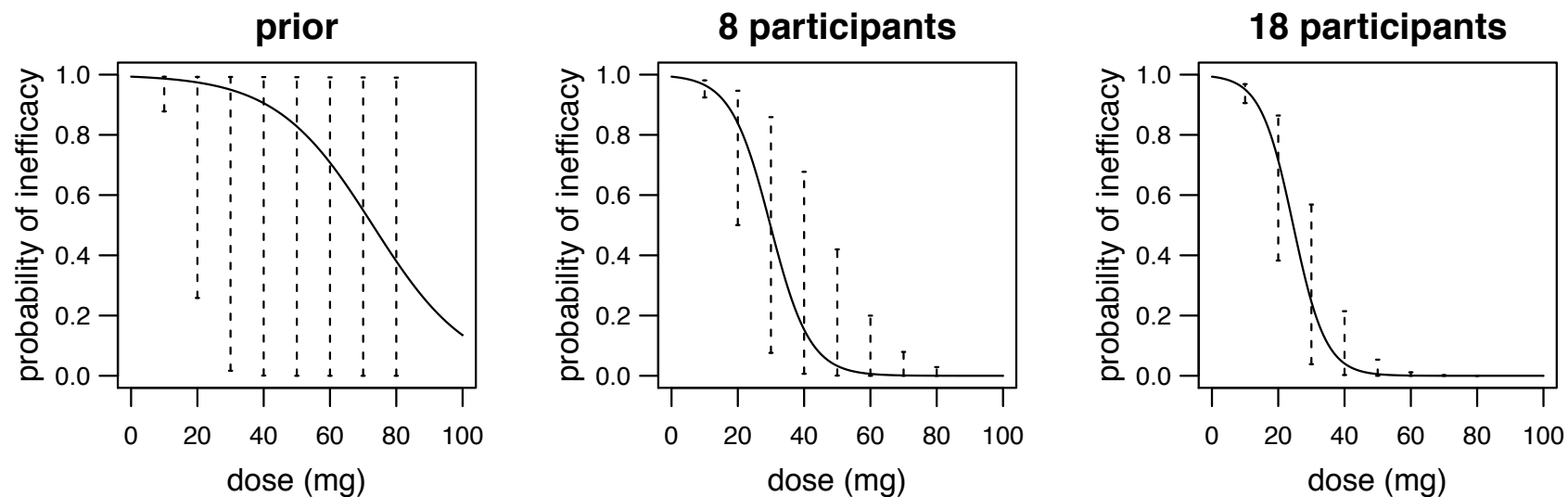
The adaptive design is considered successful if the correct MED is estimated at the end of 1,000 realisations. A dose is then chosen to be brought forward to stage 2 according to the rule-based (R) or model-based (M) approaches (**Figure 6.3**).

Figure 6.3 Illustration of scenario 1 based on 5% target inefficacy level



Crosses represent pharmacokinetic results in which the allocated dose fails to meet the endpoint, whereas black circles represent pharmacokinetic results in which the endpoint is reached. The 5% TIL is 40 mg (dashed line), therefore the aim of the adaptive and rule-based design is to select a dose at or above the TIL thus minimising the possibility of selecting a dose not achieving the pharmacokinetic endpoint. The letters 'R' and 'M' indicate the dose chosen to take forward to Stage 2 using the *rule based criteria* and *CRM* respectively. Similar figures were derived for other scenarios and at the 10% and 20% target inefficacy (*figure reproduced courtesy of Dr Alexina Mason, LSHTM*).

Figure 6.4 Change in estimated dose-inefficacy curve as participants are recruited



The figure shows the evolution in the dose-inefficacy curve as data is accrued to modify the *a priori* curve. The dose-inefficacy curve is represented as a solid line. The dashed vertical lines represent the 90% CIs for the TIL at all the possible doses. In the left plot, wide 90% CIs are observed. As data is accrued and used to adjust the *a priori* dose-inefficacy curve, the 90% CIs become narrower.

For each scenario, the probability of selecting one of the eight doses was calculated at 5%, 10% and 20% TILs. The probability of selecting one of the four doses under consideration was also calculated for the '5+5+5+5' design with the same scenarios. In addition, the simulations assessed the number of subjects allocated at each dose, the number of ineffective events and the number of ineffective doses administered. Full results are presented in **Tables 6.2 – 6.9**.

A comparison of the performance of the adaptive designs versus the '5+5+5+5' design in assigning the MED or, any higher dose, is depicted graphically in **Figure 6.5**. A positive value indicates the adaptive designs performed better than the '5+5+5+5' design in selecting the MED or any higher dose. On review, no design consistently selected the MED or higher across the seven scenarios. Within scenario 1, all designs estimated MED as 40 mg with similar probability. However, when the optimal dose was towards the upper end of the dose range, the '5+5+5+5' design selected an effective dose more reliably than the adaptive design (scenarios 2 and 3). This is most evident from the results of scenario 3 (**Table 6.4**), where the probability (p) was 0.80 that 80 mg would be selected by the '5+5+5+5' design compared with the adaptive designs where $p = 0.39-0.47$ with 5% TIL, $p = 0.28-0.31$ with 10% TIL and $p=0.12-0.44$ with 20% TIL.

Although other graphical summaries were produced to compare the performance of adaptive versus '5+5+5+5' designs (e.g. the probability of selecting the target dose or a dose 10mg higher; probability of selecting the target dose; probability of selecting the target dose or a dose 10mg higher or lower), these were not considered further as only doses which maximised that chance of a successful outcome in stage 2 were useful to the conduct of stage 2 of the clinical trial.

While the aim of the simulations was to determine the design which most reliably selected the MED (or above) across all scenarios, it was noted that the adaptive designs had other factors which could prove advantageous. For instance, the adaptive designs were able to select doses which were not covered as part of the '5+5+5+5' design (i.e. 30 mg, 50 mg, 60 mg and 70 mg). Moreover, with the exception of scenario 6, it appeared that the adaptive designs allocated more participants to effective doses than occurs with the '5+5+5+5' design; as such, this would allow more rigorous modelling of the pharmacokinetic curve at the MED and above. In particular, where all doses were effective, the adaptive design allocated the greatest number of participants to the lower dose levels (scenario 5). By contrast, where the MED was at the extreme (i.e. 80 mg) or indeed where no dose was effective, the adaptive design allocated the greatest number of participants (scenarios 3 and 4) to the higher doses, thereby allowing greater characterisation of the pharmacokinetic curve at high dose levels.

Some of the limitations in selecting the optimal dose may be offset by having a reserve of 2 participants to dose in substage 3 if D4 pharmacokinetic data from substages 1 and 2 appeared promising at a specific dose level. Indeed, within the initial proposal, it was aimed to investigate designs which may lead to more insightful exploration of the optimal dose and consequently, it was proposed to pursue the adaptive design with 5% TIL within substage 2 (**Table 6.9**).

Table 6.2: Scenario 1: Probabilities of dose selection

(MED =40 mg)

	Dose									N ineffective [†]	N below target [§]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg		
True inefficacy		0.95	0.75	0.40	0.05	0.04	0.03	0.02	0.01		
5+5+5+5											
N per dose [†]		5.00	5.00		5.00				5.00	8.82	10.00
P(selection)* - rule	0.05	0.00	0.00		0.73				0.22		
5%TIL											
N per dose [†]		2.00	2.01	0.72	7.10	2.76	0.82	0.02	2.56	4.20	4.74
P(selection)* - rule	0.02	0.00	0.00	0.07	0.48	0.20	0.12	0.01	0.09		
P(selection)* - model	0.01	0.00	0.00	0.00	0.52	0.28	0.15	0.02	0.02		
10%TIL											
N per dose [†]		2.00	2.03	1.58	8.59	1.26	0.33	0.05	2.16	4.55	5.61
P(selection)* - rule	0.02	0.00	0.00	0.01	0.56	0.18	0.03	0.01	0.20		
P(selection)* - model	0.00	0.00	0.00	0.01	0.62	0.30	0.04	0.02	0.00		
20%TIL											
N per dose [†]		2.00	2.12	4.48	6.94	0.38	0.05	0.00	2.03	5.73	8.60
P(selection)* - rule	0.01	0.00	0.00	0.01	0.69	0.06	0.00	0.00	0.22		
P(selection)* - model	0.00	0.00	0.00	0.10	0.84	0.06	0.01	0.00	0.00		

Table 6.3: Scenario 2: Probabilities of dose selection

(MED=60 mg)

	Dose									N ineffective [†]	N below target [§]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg		
True inefficacy		0.95	0.85	0.65	0.35	0.12	0.05	0.03	0.01		
5+5+5+5											
N per dose [†]		5.00	5.00		5.00				5.00	10.78	15.00
P(selection)* - rule	0.05	0.00	0.00		0.12				0.83		
5%TIL											
N per dose [†]		2.00	2.00	0.06	3.10	2.51	2.75	0.08	5.51	5.17	9.67
P(selection)* - rule	0.04	0.00	0.00	0.00	0.02	0.22	0.44	0.03	0.26		
P(selection)* - model	0.03	0.00	0.00	0.00	0.01	0.19	0.52	0.09	0.16		
10%TIL											
N per dose [†]		2.00	2.00	0.29	3.78	3.93	2.55	0.33	3.12	5.75	12.00
P(selection)* - rule	0.02	0.00	0.00	0.00	0.01	0.34	0.28	0.13	0.22		
P(selection)* - model	0.00	0.00	0.00	0.00	0.02	0.39	0.39	0.18	0.02		
20%TIL											
N per dose [†]		2.00	2.03	0.77	5.91	3.80	0.99	0.06	2.45	6.72	14.51
P(selection)* - rule	0.02	0.00	0.00	0.00	0.04	0.33	0.16	0.01	0.45		
P(selection)* - model	0.00	0.00	0.00	0.00	0.15	0.59	0.23	0.03	0.00		

Values for each trial design are means calculated over that design's set of realisations. * The probability of selecting each dose (or no dose) to take forward stage 2. † The number of subjects allocated to each dose. ‡ The number of observed ineffective events § the number of subjects treated at doses below the target dose. Mason A *et al*, 2017.

Table 6.4: Scenario 3: Probabilities of dose selection

(MED = 80 mg)

	Dose									N ineffective [†]	N below target [§]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg		
True inefficacy 5+5+5+5		0.95	0.89	0.77	0.59	0.37	0.20	0.10	0.04		
N per dose [†]		5.00	5.00		5.00				5.00	12.22	15.00
P(selection)* - rule	0.19	0.00	0.00		0.01				0.80		
5%TIL											
N per dose [†]		2.00	2.00	0.01	2.13	0.57	1.78	0.18	9.33	5.98	8.67
P(selection)* - rule	0.32	0.00	0.00	0.00	0.00	0.01	0.15	0.04	0.47		
P(selection)* - model	0.32	0.00	0.00	0.00	0.00	0.01	0.15	0.13	0.39		
10%TIL											
N per dose [†]		2.00	2.00	0.09	2.38	1.36	2.67	1.10	6.40	6.52	11.60
P(selection)* - rule	0.18	0.00	0.00	0.00	0.00	0.03	0.14	0.34	0.31		
P(selection)* - model	0.05	0.00	0.00	0.00	0.00	0.03	0.16	0.48	0.28		
20%TIL											
N per dose [†]		2.00	2.00	0.15	2.97	2.82	3.08	0.67	4.29	7.52	13.71
P(selection)* - rule	0.13	0.00	0.00	0.00	0.00	0.05	0.23	0.15	0.44		
P(selection)* - model	0.00	0.00	0.00	0.00	0.00	0.11	0.41	0.35	0.12		

Table 6.5: Scenario 4: Probabilities of dose selection

(No effective dose)

	Dose									N ineffective [†]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg	
True inefficacy 5+5+5+5		0.95	0.92	0.88	0.82	0.74	0.64	0.53	0.41	
N per dose [†]		5.00	5.00		5.00				5.00	15.49
P(selection)* - rule	0.93	0.00	0.00		0.00				0.07	
5%TIL										
N per dose [†]		2.00	2.00	0.00	2.01	0.03	0.07	0.00	11.89	10.33
P(selection)* - rule	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
P(selection)* - model	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
10%TIL										
N per dose [†]		2.00	2.00	0.00	2.03	0.07	0.26	0.04	11.60	10.40
P(selection)* - rule	0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	
P(selection)* - model	0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	
20%TIL										
N per dose [†]		2.00	2.00	0.00	2.06	0.23	0.30	0.16	11.24	10.63
P(selection)* - rule	0.95	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	
P(selection)* - model	0.89	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.09	

Values for each trial design are means calculated over that design's set of realisations. [★] The probability of selecting each dose (or no dose) to take forward to stage 2. [†] The number of subjects allocated to each dose. [‡] The number of observed ineffective events [§] the number of subjects treated at doses below the target dose. Mason *A et al*, 2016

Table 6.6: Scenario 5: Probabilities of dose selection

(All doses effective)

	Dose									N ineffective [‡]	N below target [§]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg		
True inefficacy		0.05	0.04	0.03	0.02	0.01	0.01	0.01	0.01		
5+5+5+5											
N per dose [†]		5.00	5.00		5.00				5.00	0.60	0.00
P(selection)* - rule	0.04	0.56	0.15		0.16				0.09		
5%TIL											
N per dose [†]		2.00	10.08	1.28	2.37	0.16	0.06	0.00	2.05	0.59	0.00
P(selection)* - rule	0.02	0.55	0.05	0.13	0.21	0.01	0.00	0.00	0.04		
P(selection)* - model	0.00	0.00	0.75	0.16	0.05	0.02	0.00	0.00	0.00		
10%TIL											
N per dose [†]		2.00	10.97	0.75	2.13	0.13	0.00	0.00	2.01	0.65	0.00
P(selection)* - rule	0.02	0.50	0.07	0.06	0.31	0.00	0.00	0.00	0.04		
P(selection)* - model	0.00	0.00	0.86	0.10	0.01	0.02	0.00	0.00	0.00		
20%TIL											
N per dose [†]		5.87	7.43	0.48	2.19	0.03	0.00	0.00	2.00	0.65	0.00
P(selection)* - rule	0.02	0.52	0.21	0.01	0.20	0.00	0.00	0.00	0.04		
P(selection)* - model	0.00	0.52	0.41	0.05	0.02	0.00	0.00	0.00	0.00		

Table 6.7: Scenario 6: Probabilities of dose selection

(MED = 60 mg with shallow dose-inefficacy curve)

	Dose									N ineffective [‡]	N below target [§]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg		
True inefficacy		0.50	0.40	0.30	0.20	0.10	0.05	0.04	0.03		
5+5+5+5											
N per dose [†]		5.00	5.00		5.00				5.00	5.57	15.00
P(selection)* - rule	0.14	0.00	0.02		0.29				0.56		
5%TIL											
N per dose [†]		2.00	2.48	1.91	4.71	2.67	1.28	0.05	2.89	3.94	13.78
P(selection)* - rule	0.06	0.00	0.00	0.04	0.20	0.25	0.17	0.02	0.27		
P(selection)* - model	0.02	0.00	0.00	0.06	0.21	0.33	0.27	0.08	0.04		
10%TIL											
N per dose [†]		2.00	2.86	2.71	5.05	2.17	0.71	0.07	2.42	4.35	14.79
P(selection)* - rule	0.06	0.00	0.00	0.05	0.26	0.22	0.08	0.01	0.31		
P(selection)* - model	0.00	0.00	0.01	0.13	0.30	0.34	0.16	0.04	0.02		
20%TIL											
N per dose [†]		2.01	4.08	3.78	4.86	0.91	0.18	0.03	2.15	4.88	15.64
P(selection)* - rule	0.05	0.00	0.00	0.06	0.43	0.10	0.01	0.00	0.34		
P(selection)* - model	0.00	0.00	0.04	0.32	0.38	0.20	0.04	0.01	0.00		

Values for each trial design are means calculated over that design's set of realisations. ^{*} The probability of selecting each dose (or no dose) to take forward to stage 2. [†] The number of subjects allocated to each dose. [‡] The number of observed ineffective events [§] the number of subjects treated at doses below the target dose. Mason *A et al*, 2016

Table 6.8: Scenario 7: Probabilities of dose selection

(MED = 40 mg, higher doses ineffective)

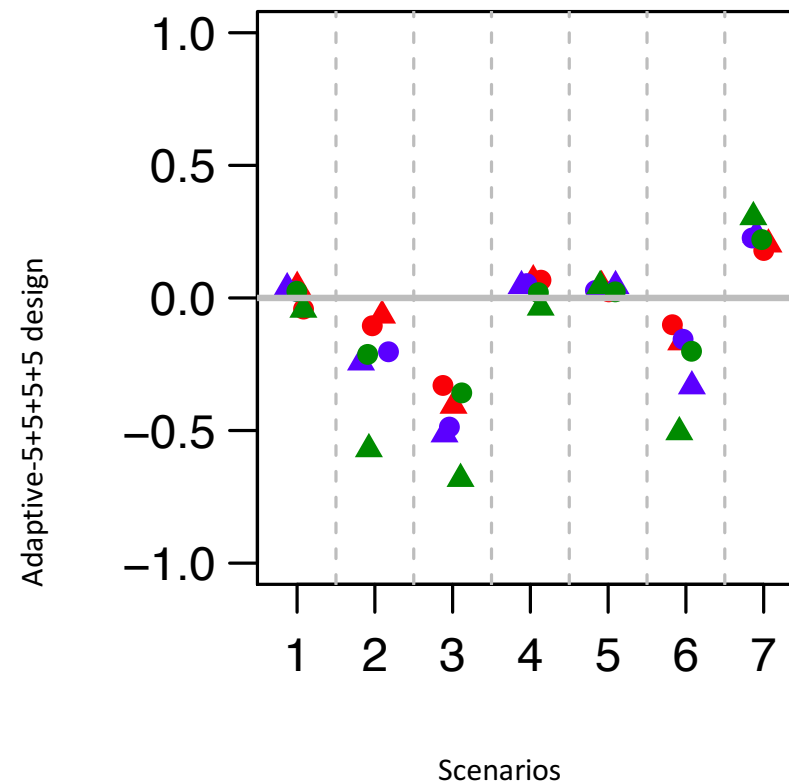
	Dose									N ineffective [‡]	N below target [§]
	None	10mg	20mg	30mg	40mg	50mg	60mg	70mg	80mg		
True inefficacy		0.95	0.75	0.40	0.05	0.10	0.18	0.32	0.50		
5+5+5+5											
N per dose [†]		5.00	5.00		5.00				5.00	11.24	10.00
P(selection)* - rule	0.97	0.00	0.00		0.02				0.00		
5%TIL											
N per dose [†]		2.00	2.00	0.20	3.17	0.70	0.26	0.04	9.63	8.56	4.20
P(selection)* - rule	0.77	0.00	0.00	0.02	0.10	0.04	0.02	0.00	0.05		
P(selection)* - model	0.77	0.00	0.00	0.00	0.11	0.06	0.03	0.01	0.01		
10%TIL											
N per dose [†]		2.00	2.01	0.45	3.71	0.34	0.14	0.03	9.33	8.48	4.46
P(selection)* - rule	0.74	0.00	0.00	0.00	0.15	0.03	0.01	0.00	0.06		
P(selection)* - model	0.73	0.00	0.00	0.00	0.17	0.07	0.01	0.01	0.00		
20%TIL											
N per dose [†]		2.00	2.08	1.09	3.31	0.51	0.26	0.07	8.69	8.56	5.16
P(selection)* - rule	0.75	0.00	0.00	0.00	0.17	0.02	0.00	0.00	0.06		
P(selection)* - model	0.64	0.00	0.00	0.02	0.23	0.04	0.03	0.01	0.02		

Values for each trial design are means calculated over that design's set of realisations. [★] The probability of selecting each dose (or no dose) to take forward stage 2. [†] The number of subjects allocated to each dose. [‡] The number of observed ineffective events [§] the number of subjects treated at doses below target dose. Mason A *et al*, 2016.

Table 6.9: Advantages and disadvantages of choosing the adaptive design over the standard ‘5+5+5+5’ design

Advantages	Neutral	Disadvantages
Adaptive design can identify the optimal dose where this lies at a different level than that tested within the 5+5+5+5 design (Scenario 2; 5%TIL and 10%TIL designs).	Very little difference in dose selected between designs for scenarios where the optimal dose is covered by the 5+5+5+5 design (Scenario 1)	Unlikely to select a dose where the maximum dose is the optimal dose (Scenario 3).
Adaptive design allocates more participants to receive the optimal dose where this also tested by the 5+5+5+5 design (Scenario 1; 5%TIL and 10%TIL designs).		
More participants are allocated to the optimal dose where this is the maximum dose (Scenario 3; 5%TIL design)		
Although less likely to select the optimal dose where this lies at the upper end of the dose range (Scenario 3), the study team can override the adaptive design. Overriding the study design would not occur with 5+5+5+5 design.		

Figure 6.5: Difference in probability between adaptive designs and '5+5+5+5' designs of choosing the minimum effective dose or higher



The figure shows a pictorial comparison between the performance of the adaptive designs vs. the 5+5+5+5 design. A positive value indicates that the adaptive design performed better in choosing the MED or a higher dose. The 5%TIL, 10%TIL and 20%TIL designs are represented by red, blue and green symbols respectively. Circles indicate rule variants and triangles model variants. Mason A *et al*, 2016.

6.5 Discussion

Comparison of the advantages and disadvantages of the CRM versus the standard '5+5+5+5' design suggest the hypothesis, an alternative clinical trial design will yield more useful clinical data compared to a standard clinical trial design, was proven.

Simulations of the scenarios proceeded on the assumption that no DLTs would occur in the trial proper. Unfortunately, the results of simulations did not clearly demonstrate that the adaptive design was superior to that of the '5+5+5+5' in estimating an effective dose across the seven scenarios. Notwithstanding, other benefits were demonstrated which influenced the choice of the adaptive design with 5% TIL. Most notably, the adaptive design was successful in allocating more participants to effective doses than occurred with the fixed allocations within the '5+5+5+5' design. The benefits of this are obvious in terms of potentially improved modelling of the pharmacokinetic data at the optimal doses. Of note, however, this did not necessarily translate into an effective dose being chosen by the model.

The '5+5+5+5' predicted effective doses more reliably than the adaptive designs as the MED increased. This is likely to have resulted from a function of the rules within the '5+5+5+5' design which stipulated, if a dose failure occurred at any level, the next highest dose would be selected. Because only four dose levels were available, were a single dose failure to occur at 40 mg, the next increment was 80 mg and this would be highly likely to be effective. Conversely, if a dose failure occurred at 40 mg within the adaptive design, the model may have estimated 50 mg as the predicted MED which would be less certain to be effective. The adaptive design allocated more participants to the MED where this was the maximum dose compared to the '5+5+5+5' design, which was fixed in terms of allocations. This highlighted the potential situation whereby if the optimal dose was at the upper extreme of the dose range, if one participant failed to meet the defined endpoint, all doses could have been ruled as ineffective. Attempts to offset some of the limitations were made by maintaining a reserve of two participants in an optional substage 3 which may be a useful consideration in the design of future ARV drug trials.

The choice of the MED is equivalent to a target trough level; within this study, a target trough level was chosen of $10 \times \text{IC}_{50}$ at day 4. For comparison, within phase 1 studies with DTG, mean trough levels after 10 days of DTG monotherapy were between $3\text{--}13 \times \text{IC}_{90}$ depending on the dose administered [174]. From a clinical perspective, the MED should be effective in all patients who are administered this. A TIL of 5% was chosen, however, as a TIL of 0% is not compatible with

the statistical design of logistic models. Given the MED was to be chosen from a discrete set of doses at 10mg intervals, a 5% TIL was considered reasonable as this would be mitigated by the rule-based and model-based approaches to dose selection and thus result in a target dose with a very low probability of inefficacy.

The uniqueness of the study is highlighted by the absence of published adaptive designs for new ARV drugs. The study design was labour intense from both a clinical and statistical perspective and clearly, requires more advanced planning and lead-in time prior to starting a study. As the era of universal ART for PLWHIV emerges however, the ability to recruit to monotherapy studies of new ARV drugs will become more challenging. Consequently, adaptive designs may yield more informative data than might be feasible given the constraints of recruitment. Moreover, as the tolerability of ARV drugs has improved, the acceptability of new drugs is highly dependent on having a low toxicity profile. It follows that future designs of adaptive trials for ARV drugs may thus be driven by similar efficacy outcomes. The dose-escalation and safety phase and simulations of the adaptive phase may consequently provide useful insights into the conduct of studies of pipeline ARV drugs.

Although DLTs are not expected with C34-PEG₄-Chol, the premise of a first-in-man study is primarily to identify toxicities associated with a new medication. Early examples of adaptive designs, notably the CRM, drew criticisms that large dose escalations could occur based on limited clinical information, thus exposing trial participants to doses in excess of the MTD [168]. The incorporation of a dose-escalation and safety phase is critical to addressing the rigour required for safety assessments in conducting first-in-man studies. By administering active drug to two participants at each dose level, coupled with period of observation between the participants, substage 1 offers the opportunity to assess sequential participants for toxicity at the same dose level before proceeding to a dose increment. Without the buffer of this dose-escalation and safety phase, the use of the D4 pharmacokinetic endpoint as the driver of the CRM could result in rapid dose escalations if no toxicities are observed at a starting dose of 10 mg. This could prove critical to the safety of the participants if unexpected adverse events occurred at lower doses. Furthermore, it could be argued that through incorporation of the dose-escalation and safety phase, the design more efficiently leads to identification of DLTs than may occur with a conventional '5+5+5+5' design. In adopting the approach, we incorporate some of the modifications previously proposed in literature such as always starting at the lowest dose increment and enrolling pairs of participants at each prescribed dose level (as opposed to relying on data from individual participants)[170].

While the design of a clinical trial is always critical in the evaluation of safety and efficacy of a drug, the development of this clinical trial presents unique challenges in both addressing safety concerns surrounding the first-in-man use of a drug and secondly in the use of an adaptive design to address efficacy.

Chapter 7

**Determination of the safety, tolerability and
pharmacokinetic profile of C34-PEG₄-Chol**

7.1 Introduction

Peptide drugs are defined in therapeutics as polymers of less than fifty amino acids and have molecular weights <10 kDa [175]. The most extensively studied peptide-based drug is undoubtedly insulin. As of 2016, the FDA had approved around seventy peptide drugs across the various therapeutic areas, most notably oncology, endocrinology and infectious diseases. It is estimated that around 10% of pipeline peptide drugs being developed are for infectious diseases [176].

Advantages of peptide drugs include generally low toxicity profiles, high potency and fewer DDIs. Aside from a small number of examples, enteral absorption is not possible due to digestion by gastric and duodenal peptidases [177]. Moreover, absorption across gastrointestinal epithelium is limited by large molecular size and hydrophilicity [176]. Peptidase inhibitors and various types of absorption enhancers have been proposed as mechanisms to overcome this [176], however thus far, subcutaneous administration is the most widespread means of administering all peptide drugs. Even via the subcutaneous route however, rapid proteolysis, antibody neutralisation and poor solubility can all limit their effects [178]. Larger peptides tend to invoke a more robust immune response causing ISRs and allergic reactions when delivered parenterally, the most widely reported example being with non-human insulins in the early era of diabetes therapeutics [179]. Moreover, subcutaneous absorption of peptides may be impeded by their large molecular weight and difficulties with transport across biologic membranes [176]. Systemic absorption may also be limited by the volume administered, local blood flow and peptide degradation. Once absorbed, high molecular weights can impact upon penetration into various compartments such that penetration may not be optimal or indeed possible, a notable example being the blood-brain barrier. Following systemic absorption, high protein binding may also limit the overall volume of distribution. Consequently, the development of clinically useful peptide drugs frequently demands novel modifications of the molecules to overcome these challenges.

Viral fusion is a critical step in the lifecycle of enveloped viruses. Besides HIV, other enveloped viruses whose entry into target cells is mediated by the presence of envelope proteins include HCV, various bunyaviruses, ebolaviruses, marburgviruses, influenzaviruses and coronaviruses [180]. While the envelope proteins which facilitate fusion are distinct in each virus, they share structural similarities and display the same mechanism of effecting viral entry i.e. the formation of a complex between HR regions to form 6-HB [180]. Although this step in the virus lifecycle is a well-established target for new drugs, only research into HIV drugs has led to a clinically approved

antiviral drug targeting this process [78, 79]. PCR now offers the ability to rapidly isolate and determine the genetic sequence of emerging viruses and by inference, determine the primary amino acid sequence of a viral envelope protein. Crystallography can help visualise the structural folding of a virus's essential proteins, and thus in turn elucidate the structural folding of the primary protein sequence [74]. Furthermore, in the era bioinformatics, it is possible to identify candidate peptides which antagonise viral entry based on genomic information. Consequently, FI peptides which prevent viral entry represent an important strategy in the development of new antiviral drugs for a range of viruses within a relatively short timeframe. So far, newer nanotechnologies have not been explored to allow oral administration of any FIs.

FIs are the only example of peptide ARV drugs. Akin to problems associated with development of other peptide drugs, the use of enfuvirtide is limited by its lack of oral bioavailability and short half-life, thus necessitating twice daily subcutaneous injections, and high rates of ISRs. Notwithstanding, enfuvirtide and other investigational FIs display low systemic toxicity and a general lack of DDIs, making them promising for further investigation as ARV agents [78, 123, 167].

Nanomedicine broadly refers to the manipulation of particles within the region of 1-100 nanometres in dimension with the aim of enhancing the pharmacokinetic and pharmacodynamic profile of a drug and in some cases, reduce toxicities and immunogenicity [181]. Examples of nanotechnologies are broad but clinically approved applications include use of liposomes, dendrimers, noncrystalline formulations and porous silicon [182]. Nano-modification of a number of HR-2-based FIs has already prolonged their plasma half-lives in both animals and humans following subcutaneous administration and fuelled a renewed interest in their development as potentially long-acting antiretroviral drugs [113, 124, 183]. The modification of the lead molecule, C34 to incorporate PEG and cholesterol (Chol) has the potential to offer insights into effects on pharmacokinetics, pharmacodynamics and tolerability in humans which could have important implications both for HIV FIs and also FIs against other viruses.

The addition of a cholesterol (Chol) moiety to C34 was proposed as a means by which to enhance antiviral potency of the drug by concentrating it within the target cell membranes where viral fusion occurs [143]. *In vitro* studies demonstrated a dramatic increase in antiviral potency and, when entered *in vivo* into rodent models, extended the drug half-life by increasing its protein binding

[143]. Based on allometric scaling from animal studies, it is expected that once-weekly subcutaneous administration in humans may be achievable with a dose range of 10-80 mg.

Similarly, addition of cholesterol to FIs targeting the entry of paraomyxoviruses (Hendra, Nipah, Parainfluenza) and more laterally with Influenza have dramatically increased potency *in vitro* compared with unmodified peptides and this was thought to be through concentrating the inhibitors within cell membranes at the site of viral entry [184, 185]. To date however, this novel nanotechnique has not been trialled in humans to assess its pharmacokinetic or pharmacodynamics effects or to assess its toxicity profile. Consequently, there is an important unmet need for clinical trials of these modified peptides which harness these novel modifications to improve bioavailability and extend circulatory half-life of peptides which extend beyond the field of HIV medicine.

Pegylation is an established method by which the dendrimer, polyethylene glycol (PEG), is covalently bound to an active molecule. Its effects include: 1) reducing immunogenicity of the compound through a 'shielding effect' from macrophages, 2) enhancing solubility of compounds thereby reducing wastage of the active compound at the active site 3) extending circulatory half-life by increasing molecular size of the molecule and thus reducing glomerular filtration and also by making the molecule more resistant to proteolysis. Although other polymers exist, the advantage of combining C34 with PEG is its safety profile in use with other medication. Indeed, PEG is already approved by the FDA in the United States and, since first used in 1990, has been labelled as 'generally regarded as safe'. PEG is eliminated unmodified either via the kidneys or in the faeces.

Based on the above, a single dose, double-blind 'first-in-man' study was planned to describe the safety, pharmacokinetic and pharmacodynamics profile of C34-PEG₄-Chol in humans. The clinical study was conducted according to the methods described in chapter 3.

Hypothesis

As the plasma concentration of C34-PEG₄-Chol increases, there will be a greater decline in plasma HIV RNA.

Aims

The aims of this chapter are therefore to:

1. Establish the safety and tolerability of C34-PEG₄-Chol across a dose range of 10-80 mg in men with HIV following a single subcutaneous dose.
2. Establish the pharmacokinetic profile of C34-PEG₄-Chol following a single subcutaneous dose in the range 10-80 mg.
3. Determine the lowest dose of C34-PEG₄-Chol which achieves a concentration 10 times above the IC₅₀ of the hardest to neutralise laboratory virus (defined according to the Minimum Effective Concentration = 25 ng/ml or 4.75 nM) at the furthest time point after administration.
4. Assess the pharmacodynamics response in terms of HIV RNA change following a single dose of C34-PEG₄-Chol in the dose range of 10-80 mg.

7.2 Results

7.2.1 Pre-clinical toxicity data

Pre-clinical toxicology studies were conducted by Charles River Laboratories, Edinburgh. Toxicology studies were conducted in 2 species: mice (n=20 at 6 mg/kg/day, n=20 at 12 mg/kg/day) and dogs (n=6 at 6 mg/kg twice weekly for 14 days, n=6 at 12 mg/kg twice weekly for 14 days followed by 1 week recovery). The subcutaneous route of administration was selected for both species. Dose levels were selected based on an attempt to produce graded responses to C34-PEG₄-Chol. A dose of 12 mg/kg/day was based on the maximal achievable dose with the formulation. No premature decedents occurred in either species. Following observation, animals were euthanised. No clinical signs, gross necropsy or histopathological findings were noted in mice up to 12 mg/kg/day; therefore the no-observed-adverse-effect-level (NOAEL) was 12 mg/kg/day. In dogs, there was no evidence of systemic toxicity with the exception of ISRs which showed reversibility following a 1-week recovery period. Based on this, NOAEL in dogs was also considered 12 mg/kg/dose.

7.2.2 Baseline characteristics

Enrolment of 28 participants (substage 1 n=16; substage 2 n=10; substage 3 n=2) was planned. Screening of participants was terminated following IDMC review results from the initial five participants dosed. Of 13 subjects screened, five were enrolled. Reasons for screen failures included deranged liver enzymes (n = 3), body mass index out of range (n=1), positive urine drug of abuse screen (n=1), CD4 count < 400 cells/ μ l (n=2), HIV RNA <10,000 copies/ml (n=1). All five participants completed 84 days of follow up. Unblinding of treatment allocation was undertaken following dosing of the fifth participant. The participants' characteristics are described in **Table 7.1**.

Table 7.1: Characteristics of participants enrolled into the clinical study

	Participant 1	Participant 2	Participant 3	Participant 4	Participant 5
Age (years)	60	28	33	19	32
Ethnicity	White	White	Mixed black/white	Black African	White
CD4 count (cells/μl)	486	496	478	407	1085
Plasma HIV RNA (copies/ml)	64763	20556	26518	12168	44283
Height	168.0	175.0	172.5	176.0	170.0
Weight	73.4	66.0	59.4	63.4	74.5
BMI	26.0	21.5	19.9	20.4	25.7
Non-HIV comorbidities	Type 2 Diabetes Mellitus	Nil	Nil	Nil	Nil
Concomitant medications	Nil	Nil	Nil	Nil	Nil
ART history	Naive	Naive	Naive	Experienced	Naive
Dose allocation	10 mg	10 mg	10 mg	10 mg	20 mg
Treatment allocation	C34-PEG ₄ -Chol	Placebo	C34-PEG ₄ -Chol	Placebo	C34-PEG ₄ -Chol

7.2.3 Safety and tolerability

Participants were assessed for local and systemic toxicities at all time points, as listed in **table 3.1**; ISRs were graded according to **table 3.3**. Adverse events and relationship to C34-PEG₄-Chol are listed in **table 7.2**.

Two grade 3 ISRs occurred in subjects administered active product (one patient receiving 10mg and one receiving 20mg C34-PEG₄-Chol). The ISRs were delayed reactions occurring several hours after drug administration (within 12 hours) with the intensity of the reaction peaking several hours to days after the administration of the product. The grade 3 criteria was met due to the size of the ISR in the participant administered 10mg C34-PEG₄-Chol (maximum size 4cm by 2cm) and degree of tenderness and pain (interfering with usual daily activities) in the participant administered 20mg C34-PEG₄-Chol. By day 7, a subcutaneous nodule was palpable. The participant was treated with simple analgesia (paracetamol) and clinical findings were confirmed following ultrasound examination at day 7 post-injection:

US Abdominal Lump report

The subcutaneous fat overlying the right rectus muscle at the level of the umbilicus is of increased echogenicity and thickness when compared to the left. This corresponds to the site of the clinically palpable lump. There is also slight oedema within the right rectus muscle itself. No focal cyst, mass or collection is demonstrated.

Impression: The appearances would be consistent with localised area of inflammation.

One participant who received active drug developed grade 1 eosinophilia (maximum eosinophil count $0.7 \times 10^9/L$), a phenomenon observed with enfuvirtide. No other systemic clinical toxicities or laboratory defined toxicities were observed.

Table 7.2: Table of adverse events

Participant	Adverse event (description)	Duration (days)	Grade	Relationship to C34-PEG₄-Chol	Action taken	Was the event serious	Expectedness
1	Injection site reaction Right umbilicus - erythema around injection site 1 hour post dosing. 12 hours: extended 15cm x 4cm but intensity of erythema lessened. Swelling at 24 hours. Erythema resolved by Day 7. Subcutaneous nodule 2 x 1cm noted Day 10	21	3 (severe)	Definitely	None	Not serious	Unexpected
1	Hyperglycaemia Glucose (non- fasting) 14.3 mmol/L. Known Type 2 Diabetes Mellitus	1	1 (mild)	None	None	Not serious	Expected

Table 7.2 (continued): Table of adverse events

Participant	Adverse event (description)	Duration (days)	Grade	Relationship to C34-PEG₄-Chol	Action taken	Was the event serious	Expectedness
1	Hyperglycaemia Grade 1. Glucose (non-fasting) 12.2 mmol/L. Known Type 2 Diabetes Mellitus	1	1 (mild)	None	None	Not serious	Expected
1	Hyponatraemia Sodium 132 mmol/L, returned to 135 mmol/L on repeat 3 days later.	4	1 (mild)	Unlikely	None	Not serious	Unexpected
3	Eosinophilia Eosinophils $0.3 \times 10^9/L$ at screening. Pre-dose eosinophils $0.7 \times 10^9/L$.	3	1 (mild)	None	None	Not serious	Unexpected

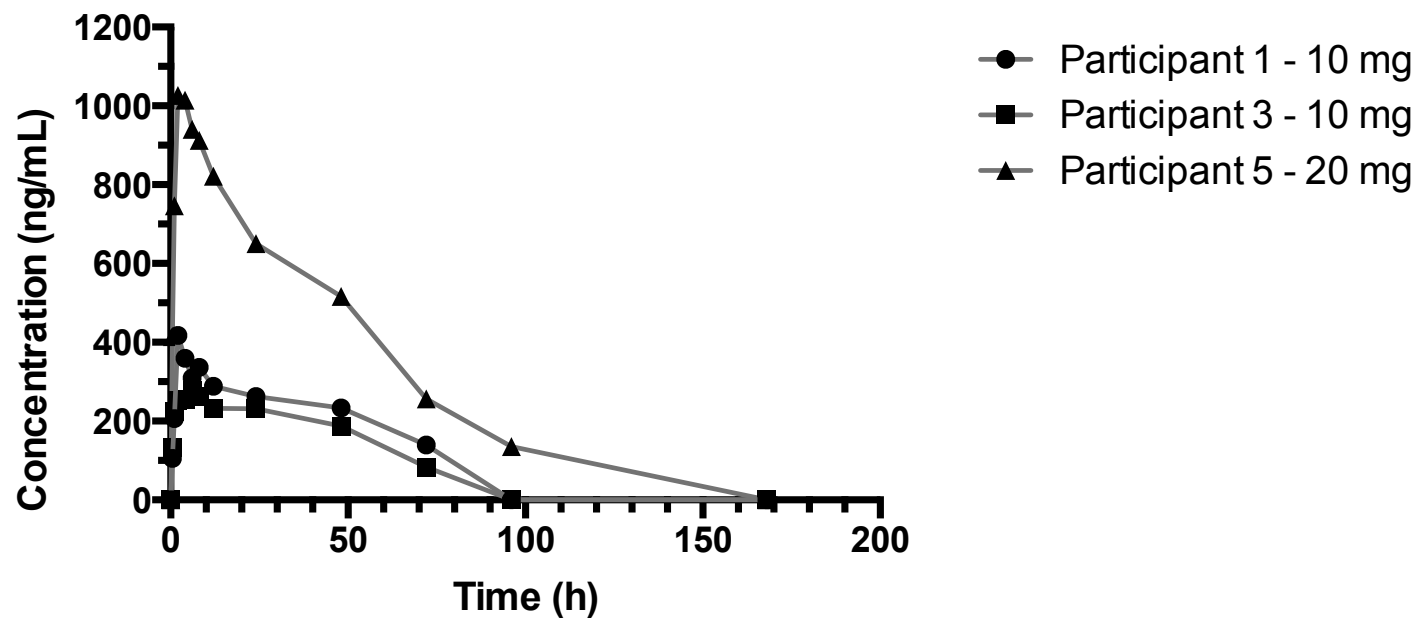
Table 7.2 (continued): Table of adverse events

Participant	Adverse event (description)	Duration (days)	Grade	Relationship to C34-PEG₄-Chol	Action taken	Was the event serious	Expectedness
3	Injection site reaction Erythema at injection site right umbilicus < 1 cm	1	1 (mild)	Probably	None	Not serious	Unexpected
3	Hyperglycaemia Grade 1. Glucose (non-fasting) 8.1 mmol/L.	1	1 (mild)	None	None	Not serious	Unexpected
4	Hot flushes Hot flushes in absence of fevers.	1	1 (mild)	None	None	Not serious	Unexpected
4	Upper respiratory tract infection Nasal congestion, pharyngitis, frontal headache.	7	1 (mild)	None	Paracetamol, phenylephedrine	Not serious	Unexpected
5	Injection site reaction Injection site reaction - tenderness, pain, erythema, pruritus	14	3 (severe)	Definitely	Paracetamol, Ultrasound abdomen	Not serious	Unexpected

7.2.4 Pharmacokinetic measurements

Plasma concentration (expressed as nanogram per millilitre, ng/mL) versus time curves following administration of C34-PEG₄-Chol to the three participants are shown in **Figure 7.1**; pharmacokinetic parameters are shown in **Table 7.3**. The $t_{1/2}$ ranged from 45-66 hours (**Figure 7.2**). The mean plasma concentrations over time (AUC_{0-t}) following administration of 10 mg, 10 mg and 20 mg to the three participants respectively were 247.317, 278.138, 505.984 ng h/ml. Plasma concentrations remained above the minimum effective concentration (25 ng/ml) for 72 hours and 96 hours in subject administered 10mg and 20mg, respectively.

Figure 7.1: C34-PEG₄-Chol pharmacokinetic time curves

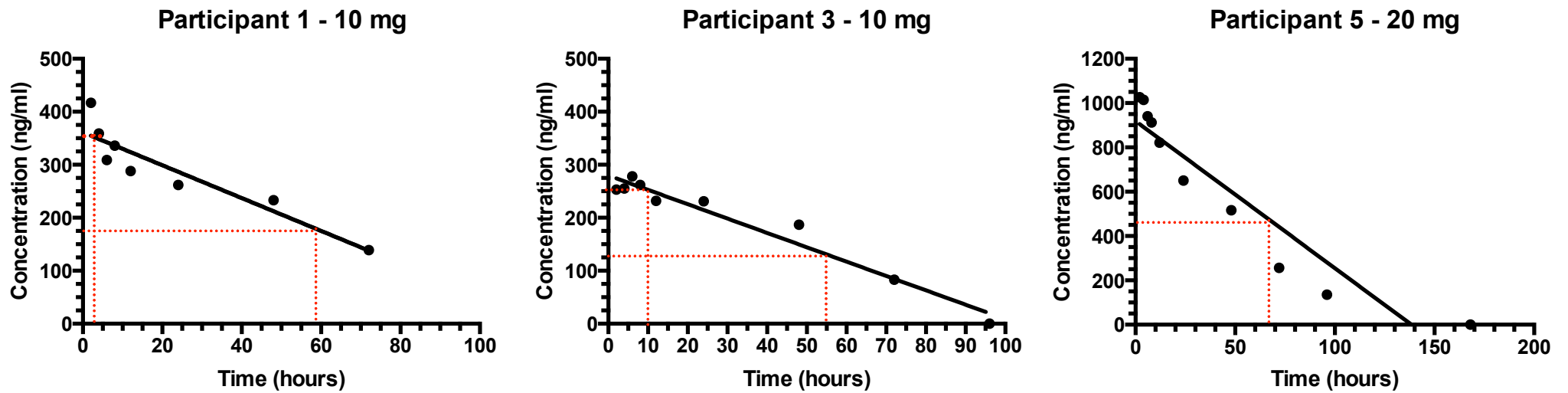


Comparison of plasma concentrations over time within the three participants administered active drug. The assay lower level of quantification (LLOQ) of drug concentration was 62.5 ng/ml. Plasma concentrations remained above the minimum effective concentration (25 ng/ml) for at least 72 hours in participants 1 and 3 and at least 96 hours in participant 5.

Table 7.3: Pharmacokinetic parameters observed in three participants dosed with C34-PEG₄-Chol

Dose	Participant 1 (Dose = 10 mg)	Participant 3 (Dose = 10 mg)	Participant 5 (Dose = 20 mg)
Maximum observed concentration $C_{\max}$ (ng/ml)	417.509	278.138	1026.720
Area under the curve (ng/h per ml)	247.317	199.472	505.984
Volume of distribution (litre)	23.98	39.5	19.47
Half-life $t_{1/2}$	56 h	45 h	66 h
Time to observed maximum concentration ($T_{\max}$)	2 h	6 h	2 h

Figure 7.2: Plasma half-life determined through linear regression



Linear regression of plasma drug concentration was undertaken; the plasma half-life was determined by the time interval for a 50% reduction in drug plasma concentration following administration. Half-life was estimated 56 h, 45 h and 66 h for participants 1, 3 and 5 respectively.

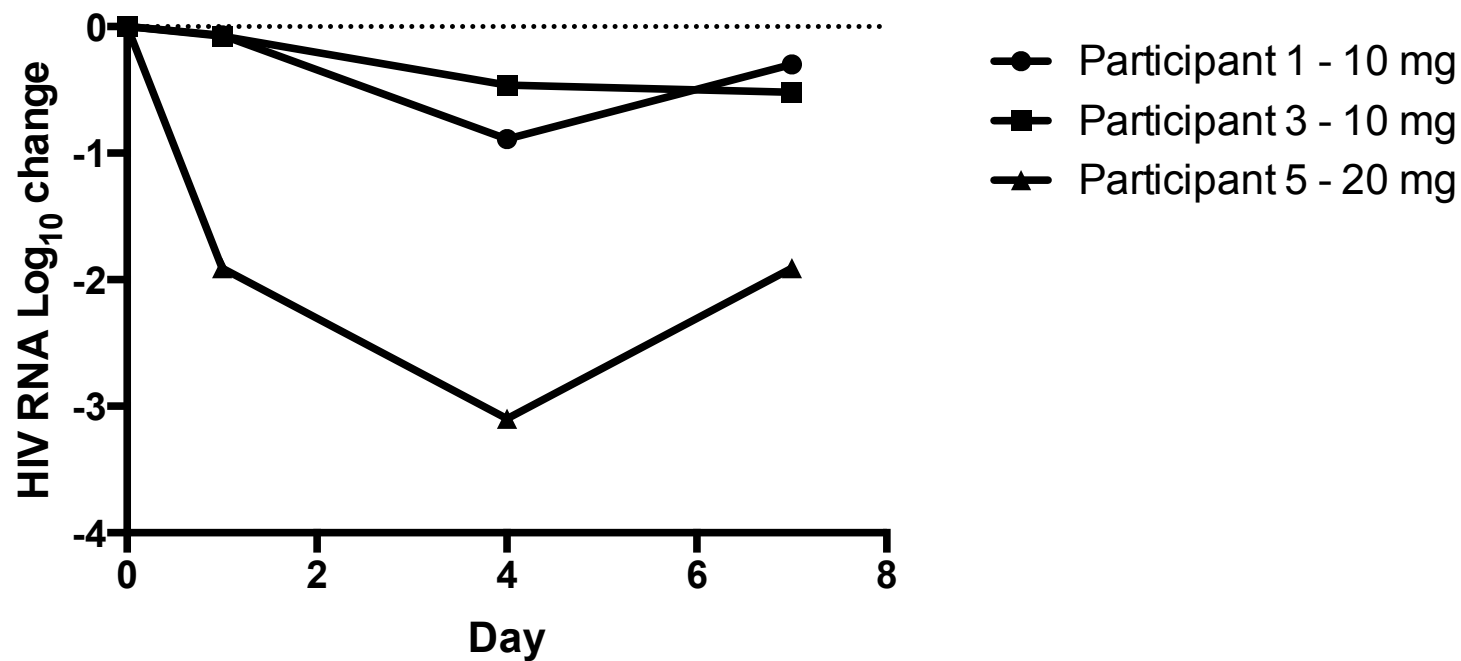
7.2.5 Measurements of antiretroviral activity

There was a significant decline in plasma HIV RNA from day 0 to day 7 of the study in all three subjects with the maximum decline in HIV RNA observed at day 4 (D4) in all (**Figure 7.3**). For the two participants who received 10 mg, a -0.89 Log_{10} and a -0.46 Log_{10} decline was observed from baseline. Although a highly significant drop in HIV RNA of -3.10 Log_{10} was observed on D4 after administration of 20 mg to participant 5, review of pre-study HIV RNA levels suggested a lower set-point HIV RNA load was normal for this participant thus mitigating the magnitude of the observed antiviral effect in this participant.

7.2.6 *In vivo* resistance

The gp41 sequence analysis of participants' virus was undertaken at three time points; day 0, 14 and 28. No evolution of mutations was observed in the gp41 sequence of the three participants who were exposed to single doses of C34-PEG₄-Chol.

Figure 7.3: Plasma HIV RNA change from baseline after exposure to a single dose of C34-PEG₄-Chol



Plasma HIV RNA changes from baseline for the three subjects exposed to a single dose of C34-PEG₄-Chol: circle, 10 mg, participant 1; square, 10 mg, participant 3; triangle, 20 mg, participant

7.3 Discussion

This is the first clinical trial on the use of the peptide, C34 and, to our knowledge, the first use of a cholesterol-tagged peptide in humans. Although results are limited to three participants, a single dose of C34-PEG₄-Chol elicited no significant systemic toxicity when participants were followed up for 84 days. A single participant displayed mild (grade 1) transient eosinophilia, which was also seen during clinic trials with enfuvirtide [78]. Administration to further participants, however was halted due to the occurrence of ISRs in two of the three participants who received the drug. In both cases, ISRs reached grade 3. In the pre-clinical studies, ISRs had not been observed in mice and were mild and reversible in dogs. In this species ISRs, albeit milder, were also observed in the control group, suggesting that the chosen formulation might have contributed to the side-effect. Doses delivered to humans were all of the same concentration (i.e. 20 mg/ml) such that participants 1 and 3 who received 10 mg, received this as a volume of 0.5 ml whilst participant 5 received a volume of 1 ml. It was considered whether ISRs could be related to extravasation of the compound due to an osmotic effect of the drug. On comparing the osmolality of C34-PEG₄-Chol with the placebo (0.9% saline), this was found to be 453 mOsm/kg vs. 308 mOsm/L. Although higher than 0.9% saline, with which no ISRs were observed, it was felt that this difference in osmolality was unlikely to explain the observed ISRs. Importantly, grading of ISRs was defined prior to the start of the study according to the categories used within the TORO 1 and 2 trials for enfuvirtide and modified from the grades defined within the Division of AIDS toxicity tables (Available at: http://rsc.tech-res.com/docs/default-source/safety/daids_ae_grading_table_v2_nov2014.pdf?sfvrsn=8). These modifications in grading were felt to be necessary as in the current era of HIV therapeutics, the occurrence of any ISR is likely to be less acceptable to patients who have options of other ARV drugs with low toxicity profiles. In addition, although high rates of ISRs with enfuvirtide did not prove an impediment to its use, this was largely due to enfuvirtide forming part of ART 'salvage' regimens in the pre-integrase inhibitor era. Interestingly, despite its structural similarity to C34, ISRs were not observed with any significant frequency with SFT in a phase 1 trial of 60 HIV-negative participants and only around 7% of participants in a phase 2 study [124]. In 'first in human' studies with rilpivirine-LA nanosuspensions (dissolved with a surfactant, poloxamer 338), more ISRs were observed following subcutaneous injections compared with intramuscular injections. In addition, intramuscular injections tended to be better tolerated in gluteal muscles compared with the deltoid and these results were replicated in a further phase 1 safety study [50]. Within its present formulation however, to consider a further trial of C34-PEG₄-Chol with intramuscular delivery, large volume injections would be required above 20 mg or alternatively, more than one injection

to achieve prolonged therapeutic effect. Moreover, direct inference cannot be made with rilpivirine-LA given the lack of similarity between the two compounds.

In the two participants who received 10 mg of C34-PEG₄-Chol, the lower limit of quantification (LLOQ, 62.5 ng/ml) was reached after 72 hours while in the participant who received 20 mg, this was reached after 96 hours. This concentration is still 25-770 fold the IC₅₀ observed *in vitro* on several viral strains and notably, it coincides with the threshold (12.8 nM; 67 ng/mL) which prevents growth of resistant virus *in vitro*. The primary endpoint of the study was to determine the minimum effective dose (MED) as defined by the lowest dose which achieves a plasma levels 10 x time above the IC₅₀ (25 ng/ml or 4.75 nM) at the furthest time point after administration; from these limited results, the PK curves suggest that 72 hours after administration in participants 1 and 3 that plasma levels remain above 25 ng/ml whilst after 20 mg, plasma levels remain above this threshold up to 96 hours. Extrapolating these results to higher doses, up to the intended maximum of 80 mg, strongly suggests that once-weekly use could be possible, should no local or systemic toxicities have been observed.

The preliminary pharmacodynamic data showed $\geq 0.5 \text{ Log}_{10}$ reduction in plasma HIV RNA in all three participants after a single dose at the lower end (10-20 mg) of the intended dose range (10-80 mg). Notably, rebound of viral load was measured only after 96 hours, which is consistent with a plasma concentration of C34-PEG₄-Chol above the IC₉₀ for longer than our assay could measure.

Whilst use further *in vivo* evaluation of C34-PEG₄-Chol is not planned at this time, the limited pharmacokinetic evaluation of the compound as cholesterol tagged peptide augurs well for further investigation; in particular, use of peptide-based FIs remain under investigation for numerous viruses where no therapies currently exist. As such, if the cholesterol tag is responsible for the observed ISRs, this side-effect may well be less relevant in the context of a situation where no alternative therapy exists and where a cholesterol tag optimises the pharmacokinetic profile of a drug.

Chapter 8

General discussion

8.1 Introduction

The primary aims of ART are to reduce morbidity and mortality associated with progressive immunosuppression and immune dysfunction associated with HIV infection. Arguably, with the current repertoire of ARV drugs, this has now largely been achieved. The results of the HPTN-052 study have also conclusively demonstrated the public health benefits of ART such that TasP is now a well-accepted indication for treatment initiation. Moreover, with results of the START study showing a reduction in non-AIDS morbidity associated with early ART coupled with general improvements in tolerability and simplicity of regimens, there are now clear benefits to recommend universal ART regardless of CD4 count. With the current array of well-tolerated and simple ART regimens, it could be argued that the margin for improvement on the current repertoire is small.

There are, however, persistent unresolved issues with current drugs. With lifelong use of ART, there remain concerns about cumulative toxicities, resistance and side effects. Furthermore, the ageing cohort of PLWHIV now means that the management of DDIs with non-HIV medications is a significant clinical problem. In addition, problems around polypharmacy with older PLWHIV have challenged pharmaceuticals to invest in research into long-acting ARV drugs. Certainly, while there is now a long list of ARV drugs, management of patients is now dictated by a relatively small core of drugs from across five of the six current classes of drug. Consequently, there remains a need for new drugs but with a shift in focus to develop agents of equivalent efficacy, improved tolerability, low toxicity profiles, no DDIs and which can be delivered as part of a simple regimen.

This thesis examined a novel ARV drug from its development to its first-in-man use. In doing so, four hypotheses were addressed:

8.2 Discussion of Hypothesis 1: In the presence of residual HIV viral replication, drug pressure will result in the selection of mutations in the HR-1 region of gp41.

A single point mutation (A71T) was identified within the HR-1 domain of gp41 following successive passages with C34-PEG₄-Chol, thus proving the first hypothesis. In addition, accessory mutations were observed within the HR-2 domain of gp41 (E136G and E148K).

Understanding the resistance profile associated with the drug was considered a core development issue, which until the point of clinical trial development, had not been addressed. It is recognised that the successful use of any ARV drug is dependent on understanding how mutations in the primary sequence of the virus may affect susceptibility to the drug. As the lead molecule, C34,

overlaps by around two-thirds with enfuvirtide, it was initially expected that a similar resistance patterns would emerge. Although, the findings were somewhat unexpected, review of the interface between C34 and the HR-1 domains of gp41 differs from that of enfuvirtide. Specifically, C34 tends to interact with the PBD of HR-1 whereas the activity of enfuvirtide is upstream and interacts with the LBD. Another possibility is that the superior antiviral potency of C34-PEG₄-Chol constitutes a “resistance capacitor”, a reserve of binding energy that provides a strong genetic barrier to resistance [186, 187]. Indeed, mapping of the A71T and the compensatory mutations E136G and E148K onto the post-fusion structure of gp41 (**Figure 4.10**) suggests that resistance is not due to increased stability of the 6-helix bundle.

Limitations are recognised in the proof of this hypothesis in that only a single mutation was identified within the HR-1 region. In the first instance, multiple parallel cultures were not undertaken; this approach may have led in a greater diversity of mutations being recognised. Repeat cultures were undertaken with smaller increments between escalations in the drug concentrations from one passage to the next in an attempt to select a dominant viral species more effectively. These cultures, however, were started from a viral culture which had already had drug exposure, and as such the resistance pathway for this viral line was likely already selected. Secondly, it is probable that while other mutations were selected during *in vitro* passage work, these may not have been sequenced due to their occurrence below the 20% cut-off level for detection by Sanger sequencing.

8.3 Discussion of Hypothesis 2: Mutations occurring in the presence of C34-PEG₄-Chol will confer reduced susceptibility of HIV to the drug.

Subsequently, it was aimed to test the second hypothesis by determining the phenotypic effects of mutations on susceptibility to the drug. This was done by undertaking site-directed mutagenesis to introduce the A71T mutation into the laboratory HIV strain, NL4-3. Thereafter, the mutated virus was incubated in a susceptible cell line with various drug concentrations and followed by estimation of a viral entry using a firefly luciferase assay to demonstrate luminescence. The methodology was successful in confirming the ability of the A71T mutation to confer resistance at a modest level. Unfortunately, technical and time constraints prohibited further exploration of the accessory mutations. Nonetheless, their presence in viral isolates, which were growing at high concentrations of C34-PEG₄-Chol, lends weight to the assumption that these accessory mutations were able to augment resistance to the drug. Indeed, the findings of accessory mutations have been demonstrated with other HR-2-based FIs and their effects have invariably shown enhanced viral

resistance by several fold when they occur with a primary resistance mutation. Notwithstanding, by demonstrating the effect of A71T on drug susceptibility, the second hypothesis of this thesis was proven.

8.4 Discussion of Hypothesis 3: Alternative clinical trial designs will yield more useful clinical data compared to a standard clinical trial design.

Adaptive clinical trial designs were explored for the first-in-man study. Since the first description of the CRM in 1990, use of modified versions of CRMs have gained acceptance in other fields of medicine but so far, there are no reports of use in HIV therapeutics. As highlighted previously, the universal use of ART is likely to limit the future ability to efficiently perform monotherapy studies of new drugs as we enter the era where the aim is to start almost all patients on ART. Thus, there is a need to consider innovative approaches maximise output of useful data with limited cohorts of patients. The use of a pharmacokinetic guided endpoint guided by efficacy is novel within a phase 1 HIV study. Within oncology studies, the aim is usually to identify the MTD. Within our approach, we sought to identify the MED, defined according to the lowest dose which was able to achieve a plasma level 10 x above the IC_{50} of the drug at D4 post administration. Given that there is high tolerability associated with most contemporary ARV drugs, the idea of attempting to determine the maximum ‘tolerated’ dose with any new drug seems redundant. Of course, efficacy may be defined in numerous different ways, however, our pharmacokinetic definition seemed appropriate given the desire to investigate a potentially long-acting ARV drug.

By adopting a hybrid approach to the study design with a dose-escalation lead-in, it was possible to maintain robust assessments of safety, an approach which may prove useful in design of future adaptive trials for ARV drugs. Moreover, the simulations identified that the statistical algorithms were likely to lead to the allocation of more participants to therapeutic doses as the model ‘learns’ to modify the dose-inefficacy curves. With these results, it is possible to accept the third hypothesis of this thesis, that alternative clinical trial designs will yield more useful clinical data compared to a standard clinical trial design.

8.5 Discussion of Hypothesis 4: As the plasma concentration of C34-PEG₄-Chol increases, there will be a greater decline in plasma HIV RNA.

The fourth hypothesis was tested by exploring the dose-response relationship during the course of the clinical trial. Within the trial, participants were administered a single dose of C34-PEG₄-Chol, commencing at the lowest dose of 10 mg before an increment to 20 mg. Significant ISRs were

encountered which limited the trial to three participants. While ISRs were encountered with enfuvirtide and other HR-2-based FIs, the significance of the reaction was unexpected in terms of severity. Additionally, with enfuvirtide, there appeared to be a relationship between ISRs and the cumulative number of injections (i.e. ISRs became more common with more doses). Although a very significant decline in D4 HIV RNA was observed with the participant who received 20 mg compared with the participants who received 10 mg, review of his previous HIV RNA loads before entry suggested that his set point was lower than what was observed in screening, thus mitigating the effect seen. Thus, it is not possible to conclude that the fourth hypothesis (i.e. as the plasma concentration of C34-PEG₄-Chol increases, there will be a greater decline in plasma HIV RNA) was proven.

Nevertheless, pharmacokinetic data for the three participants who received C34-PEG₄-Chol was promising in terms of projecting that, had higher doses of the drug been tolerated, it may indeed have been possible to dose the drug twice a week, or, less frequently. Moreover, although significant ISRs were observed with subcutaneous dosing, no systemic toxicities were observed, underlining experience with other HR-2-based FIs which have demonstrated few, or no, toxicities (aside from ISRs). Although it was not possible to prove the fourth hypothesis, it is intuitive that a higher dose of drug, if tolerated, would lead to a greater decline in HIV RNA. Studies with rilpivirine-LA and cabotegravir suggest that intramuscular injections may be better tolerated. Also, although ISRs were extensively reported with both drugs, their occurrence in clinical trials did not dissuade participants from potential future use. While no future clinical trials with C34-PEG₄-Chol are planned, there remains merit in future exploratory studies of long acting HR-2-based FIs.

8.6 Future strategies for trials of long-acting injectable antiretroviral drugs

Future strategies for the delivery of ART with long-acting injectable agents have yet to be elucidated and are likely to vary according to the agents in use. For example, the development of rilpivirine-LA and cabotegravir have offered an important insight into how such regimens may proceed: importantly, both drugs have oral formulations which allowed a lead-in oral phase within the LATTE study to assess tolerability of the agents over a prolonged period before administering both drugs parentally. By contrast, ABT does not have oral bioavailability and thus can only be delivered by injection; as such, there is no opportunity to assess an individual's tolerance of ABT prior to first administration via injection. Consequently, it could be argued that agents which do not have an oral formulation with which to trial an oral lead-in phase prior to delivery of injection, would require an even cleaner toxicity profile. Initiation of a long-acting ARV drugs at a low dose

could allow assessment for adverse reactions or toxicity before introducing the full treatment dose. Such an approach however would have a high likelihood of selecting resistance associated mutations. An “induction-maintenance” approach, however, may allow this where a lead-in period with standard oral cART to ensure viral suppression is achieved is followed by administration of the injectable ARV drug at a low dose to assess its tolerability before escalating to full therapeutic dosing. After ensuring tolerability of the injectable drug, the regimen could subsequently be simplified by withdrawal of some of the oral agents.

8.7 Future directions with cholesterol-endowed peptide drugs

While the use of HR-2-based FIs, *per se*, are not novel, their adaptation with the addition of cholesterol moieties is. It is important to recognise the value of the results obtained beyond the field of HIV medicine. In particular, FI peptides may, in future, play an important role in the armamentarium of antiviral agents against a range of other viral infections which use similar mechanisms to gain entry into the target cells of hosts. So far, these drugs have not yet been entered into clinical trials. It should be remembered, however, that although significant ISRs were observed with the use of C34-PEG₄-Chol, modification with cholesterol was shown to markedly enhance the antiviral efficacy of the drug *in vitro*. Consequently, although C34-PEG₄-Chol was tolerated poorly with ISRs, this side effect may be more acceptable with other viral infections for which there are limited therapeutic options. As such, this work provides a basis on which to seek and trial the use of cholesterol-endowed FIs for use in other viral infections.

The limitations of C34-PEG₄-Chol notwithstanding, HR-2-based FIs merit further investigation as therapeutic options. This is especially true in viral infections which use similar mechanisms of action for cell entry, but for which no therapeutic options yet exist. Despite ISRs, cholesterolisation of these molecules is of continued interest in enhancing potency and circulatory half-life of peptides. Their malleability and low systemic toxicity profile warrants their investigation within HIV as long-acting agents. Moreover, the investigation of the resistance pathways provides valuable insights, not only into the management of resistance, but also in understanding the molecular properties of the gp41 subdomains which can inform decisions on future lead molecules for investigation.

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