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Cancer gene therapy with targeted adenoviruses

Houria Bachtarzi, Mark Stevenson & Kerry Fisher†
†*University of Oxford, Department of Clinical Pharmacology, Old Road Campus Research Building, OX3 7DQ, Oxford, UK*

Background: Clinical experience with adenovirus vectors has highlighted the need for improved delivery and targeting. *Objective*: This manuscript aims to provide an overview of the techniques currently under development for improving adenovirus delivery to malignant cells *in vivo*. *Methods*: Primary research articles reporting improvements in adenoviral gene delivery are described. Strategies include genetic modification of viral coat proteins, non-genetic modifications including polymer encapsulation approaches and pharmacological interventions. *Results/conclusion*: Reprogramming adenovirus tropism *in vitro* has been convincingly demonstrated using a range of genetic and physical strategies. These studies have provided new insights into our understanding of virology and the field is progressing. However, there are still some limitations that need special consideration before adenovirus-targeted cancer gene therapy emerges as a routine treatment in the clinical setting.

Keywords: adenovirus, genetic modifications, gene therapy, polymer-coating, transductional targeting

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1. Introduction

Advanced metastatic cancer remains a very difficult disease to manage despite recent advances in care and treatment. In the late stage, patients are typically burdened with extensive tumour nodules that have acquired multiple genetic changes enabling them to resist the activities of drugs, suppress the immune system and subjugate normal cells to provide nutrients and extracellular matrix. Fighting such a complex disease with highly selective, mono-functional drugs has obvious limitations, while broadly toxic chemotherapy agents produce ever-decreasing returns for the discomfort they cause.

Virotherapy, as a concept, provides a compelling alternative to small drugs for the treatment of complex diseases. In principle, gene-based medicines are capable of multi-modal intervention that can be customised and tailored for each specific disease. For example, a cancer selective oncolytic virus may be further augmented by 'arming' with antivascular agents or immunostimulatory molecules.

The ability to precisely control therapeutic gene expression using promoters and the potential for amplification through processes of transcription, translation and replication has attracted a great number of scientists and clinicians to consider gene-based and oncolytic therapies.

For the treatment of cancer, adenovirus has been the vector of choice because of its ability to infect a wide variety of cell types with high efficiency. Most importantly, adenovirus is a robust vector that can be manufactured to very high titres and stringently purified.

Over the last 15 years an astonishing range of gene therapies have been devised, not least for cancer, demonstrating remarkable utility in experimental models. Unfortunately clinical outcomes have not lived up to expectations (with a few notable exceptions) and this has had a substantial deleterious impact on the

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field. For some time it has been recognised that the main limitation of gene-based medicine is delivery of a sufficient number of viral particles to target cells, rather than a problem with the underlying molecular strategies. The relatively large size of virus particles restricts penetration through tissues, while the innate and adaptive immune systems frustrate systemic delivery. Accordingly many protocols involving therapeutic viruses have involved local and regional delivery. However, this approach is only likely to be useful for niche indications where a cancer is locally confined and not curable by existing treatments such as surgery or radiotherapy. The majority of cancer patients present with disseminated cancer and this usually requires drugs to be delivered systemically.

Recognising the benefits of adenovirus as a versatile platform, many researchers have sought to adapt, evolve and make improvements rather than switching to alternative but less well-characterised viruses. In this review we describe a range of published strategies designed to improve adenoviral targeting and overcome some of the barriers that restrict effective delivery in the patients. The merits of each of technique will be discussed within the context of what the authors believe are the most important criteria of a successful vector for cancer therapy.

These are exciting times for targeted gene delivery with the first clinical trial of a tropism modified conditionally replicating adenovirus vector (HAdV-5-D24RGD) currently underway in Birmingham, Alabama [1].

2. The importance of detargeting

Infection of non-target cells or particle scavenging by macrophages is no so much an issue for toxicity with adenovirus vectors but more of a concern for vector loss. Cells of the reticular endothelium system (RES) rapidly remove particles from the bloodstream [2], leaving insufficient material to reach the desired target. The RES is particularly active in the liver, where particles are first filtered through sinusoidal endothelium into the space of Disse and then exposed to large number of specialist particle scavenging macrophages called Kupffer cells. Given that the liver receives approximately 30% of the total blood volume per circulation, it is easy to see why virus circulation kinetics are so poor. In comparison, the blood flow to tumours is very poor and inconsistent [3]. Under these conditions, 'targeting' alone is unlikely to provide any appreciable benefit unless the rapid clearance and inactivation of particles are first addressed.

3. Avoiding unwanted interactions with blood components

The impact of neutralising antibodies on adenoviral vectors has been well understood and characterised. However, there are many other interactions that could also have a negative impact on the ability of virus particles to circulate or infect target cells. Erythrocytes, for example, will bind HAdV-5 with sufficient strength to prevent subsequent infection of tumour cells [4]. Other blood components, such as clotting factors [5], complement [6] and non-neutralising antibodies may also impact on virus kinetics and availability. Ideally all unwanted interactions should be identified and avoided, while any potentially beneficial associations should be purposefully exploited.

4. The challenges for active targeting *in vivo*

Targeting cells surface molecules *in vivo* can be readily achieved with monoclonal antibodies. Here, small changes to key amino acid sequences can have a significant impact on antibody distribution and function. Such minimal changes to adenovirus surface proteins are unlikely to have such a dramatic effect *in vivo* due to the relatively large size and poor kinetics of virus particles. Following i.v. administration, the fate of particles is largely governed by physical constraints such as blood flow, endothelial tight junctions and filtration in the liver, and there is little opportunity for targeting strategies to be influential.

Should extended plasma kinetics be achieved, it would then be possible to exploit the enhanced permeability and retention (EPR) effect [7]. This phenomenon can occur in tumours that have leaky endothelium, where particles are able to leave the bloodstream by convection and become trapped and concentrated in the stroma. The EPR effect is often exploited for the delivery of high molecular weight drugs [8] and liposomes [9] and should also be applicable for virus particles. Once a virus particle has entered the tumour stroma, targeting elements directed against tumour cells then have a chance to find their targets. Although an 'active' targeting strategy directed towards a tumour cell may increase the probability of virus particle infection once in the stroma, they are unlikely to have a substantial impact on the overall quantity of particles entering or remaining in the tumour.

5. The lack of suitable animal models

Many of the interactions that are likely to have an important impact on virus behaviour in humans do not occur in mice. For example, erythrocyte binding and monocyte infection are not observed in mice models [4], rendering them unsuitable for systemic studies with HAdV-5. Even within the same species significant differences in adenovirus distribution have been observed based on subtle differences in the physical anatomy of the liver [10]. The lack of suitable small animal models that reflect all of the important vector–host interactions of humans has frustrated the successful translation of therapies from the laboratory to the clinic. To improve our understanding of vector behaviour in the laboratory, greater attention

should be made to human fluid and tissue samples, where possible. For agents designed for systemic delivery, a simple vector survival assay in whole human blood would be a valuable laboratory test, yet it is rarely performed or published.

6. Choice of adenoviral vector serotypes in cancer therapy

Historically HAdV-5 has been the vector of choice as probably the most well understood and widely available serotype in research laboratories. Recognising that this virus may not be the most appropriate starting point for therapeutic applications, many researchers have looked closely at other serotypes. In 2003 Vogels *et al.* published one of the most useful papers in the field of adenovirus delivery, a comprehensive assessment of neutralising antibody titers in human serum against a range of adenoviral serotypes [11]. This work allowed the identification of some group B viruses such as HAdV-11 and HAdV-35 that have the lowest frequency of neutralising antibodies in the European population. Furthermore, these viruses are capable of infecting cells via the complement regulatory protein CD46 [12] that shows a more favourable tropism for tumour cells [13].

The use of these alternative serotypes could provide a significant benefit over HAdV-5 where there has been increasing concern regarding the downregulation of the Coxsackie Adenovirus Receptor (CAR), the primary receptor for HAdV-5 in many advanced cancers [14-18]. Although it is unclear to what extent CAR makes a difference to infection *in vivo* following work describing the importance of heparin sulfate binding [19] or clotting factor associations 4 for HAdV-5 infection *in vivo*.

When chosen for specific applications, alternative vectors to HAdV-5 can demonstrate some clear advantages; however it is possible that some drawbacks may arise when used in the clinics. Binding CD46 may not be entirely beneficial for non-local delivery protocols, since almost all nucleated cells express some level of this essential protein [20].

Selecting viruses with low sera prevalence is only going to provide a window of opportunity before the inevitable induction of an adaptive response. Some may argue that this will be sufficient for an oncolytic vector capable of replicating through a tumour mass. However, knowledge of tumour structure, the intermittent blood supply [21] and resistance of hypoxic regions to virotherapy [22] all indicate that multiple doses will be required.

7. Chimeric vectors

Rather than switching completely to an alternative vector, some research groups have investigated combining desirable characteristics from different viruses. For example, switching fibres between serotypes can provide a new tropism for established group C vectors. Yu *et al.* reported an increased infection of oesophageal and oral carcinoma cells compared with the unmodified virus after substituting HAdV-5 fibres with those from HAdV-11 or HAdV-35 [23]. Similarly, the use of HAdV-3 fibres on HAdV-5 backbone was shown to be particularly effective for targeting ovarian cancer, squamous cell carcinoma of the head and neck and B cell lymphomas [24-26].

Incorporating alternative fibres into existing oncolytic vectors allows targeting gains to be compounded over sequential rounds of replication. Reddy *et al.* demonstrated that a fibre chimeric Ad vector expressing the E1 A gene under the control of the tumour-selective promoter E2F-1 led to a greater therapeutic response, with 2- to 576-fold increased killing of melanoma and head and neck cancer (HNC) cells. A stronger antitumour effect and improved survival were also demonstrated *in vivo* upon intratumoural injections into melanoma and HNC xenografts [27].

A natural extension of this work was the identification of optimised oncolytic viruses through a process of forced evolution [28]. In this study Kuhn *et al.* first encouraged recombination by super-infecting tumour cells with multiple Ad serotypes from subgroups B – F. Progeny virions were then subject to 18 rounds of increasingly stringent selections in order to isolate chimeras with enhanced tumour lytic properties. Given that we know little about many of the rare adenovirus serotypes, such viruses are unlikely to be identified by rational design.

Constructing designer vectors from the existing repertoire of adenovirus serotypes has shown some very clear benefits, and new combinations are likely to be published in the future. However, the suitability of these vectors for application other than local administration is likely to be limited. For example, it has been shown that switching structural proteins has little effect on rapid 'particle-based' clearance of viruses to the liver [29]. These vectors are also likely to be vulnerable to neutralising antibodies that will inevitably be produced against them.

8. Selective modification of amino acid sequences in capsid proteins

8.1 Fibre modifications

Genetic modification of specific amino acid residues within the fibre knob has been the subject of many investigations [30-33]. Ablating the natural tropism of the virus *in vitro* has been achieved by minimal substitution in the AB, DE or FG loop of the virus fibre knob domain [33]. However, Bayo-Puxan *et al.* have shown that additional mutations of the KKTK motif of the fibre shaft domain and penton base RGD mutations are also beneficial to achieve tropism ablation [34]. *In vivo*, the tropism profile of these CAR-binding mutated vectors revealed no significant difference from the wild-type HAdV-5 virus [30,35]. More recently, a CR2 (constant region 2)-mutated conditionally replicating adenovirus was genetically retargeted by incorporating a poly

(L-lysine) oligopeptide at the C-terminal end of its fibre. This engineered virus was shown to exhibit efficient killing of breast cancer cells *in vitro*, but when administered intravenously was not superior to an isogenic virus carrying wild-type fibres [36].

8.2 Hexon and penton base engineering

The hypervariable region loop of hexons and selective regions of penton base have been subject of intense investigation for the purposes of improved gene delivery. Proof of concept was first demonstrated by Vigne *et al.* who were able to insert an αv integrin binding peptide (RGD) into the hypervariable region 5 of HAdV-5 and demonstrate improved transfection of cells refractory to HAdV-5 infection, such as human vascular smooth muscle cells [37].

The crucial role of penton base in interacting with αv β3/β5 integrins and consequently mediating virus internalisation and gene transduction prompted additional investigation of this protein for its efficiency to hold targeting ligands. Einfeld *et al.* have previously reported a retargeted adenoviral vector with haemagglutinin, a peptide that was genetically linked to the penton base and shown to have no effect on overall virus structural integrity and function [38]. However, retargeting strategies at these sites seem to be restricted to small size ligands [37,39,40], thus limiting their applicability in gene therapy.

8.3 Protein IX as a novel site for genetic attachment of retargeting ligands

Protein IX is a minor protein located on the surface of mature virus capsid. The N-terminus is embedded within the group-of-nine hexons, which form the facets of the adenovirus icosahedral structure; the C-terminal end exposed at the surface [41,42], representing a potential point of attachment for retargeting ligands. Vellinga *et al.* investigated the incorporation of alpha-helical spaces of different lengths, linking the RGD motif to the C-terminus of protein IX, showing improved transduction of some CAR-negative cell lines, including the mouse haemangioendothelioma cell line Eoma [43]. Similarly, Dmitriev *et al.* reported incorporating a Flag (DYKDDDDK) octapeptide joining a sequence of eight lysine residues or a poly (L-lysine) sequence to the C-terminus of protein IX. This modified virus was able to restore infectivity through the heparan sulfate receptor in cells with low CAR expression levels [44].

8.4 'Platform' targeting strategies

Incorporation of the Fc binding domain from *Staphylococcus aureus* protein A or *Streptococcus aureus* protein G provides a platform for evaluating a range of targeting possibilities with the same core vector. Henning *et al.* have clearly demonstrated the feasibility of this approach, opening up possibilities of using ligands including antibodies whose functional activity is compromised when genetically incorporated within the vector capsid [45,46]. Further work identified that not all antibody-receptor targeting combinations were suitable for all cells [47] and that additional factors were required post-internalisation to disrupt the endosome.

A parallel approach was developed by Parrott *et al.*, who engineered a 71 amino acid biotin acceptor peptide isolated from *Propionibacterium shermanii*, into the C-termini of the fibre [48]. The vectors were then retargeted via tetrameric avidin molecules taking advantage of the strong biotin–avidin affinity. Subsequently the same group went on to generate a virus with the biotin acceptor sequence fused to protein IX. This format offers more copies of biotin and was easier to purify by affinity chromatography. Interestingly only fibre modified, but not protein IX modified, virus particles were effectively retargeted with biotinylated antibodies against CD71. However, protein IX modified vectors could be retargeted with native transferrin, the ligand for CD71 [49].

Both of these techniques (Fc and biotin incorporation) enable candidate targeting ligands to be screened rapidly *in vitro*. They provide a convenient method for purification of virus particles from complex environments.

Overall, the selective modification of amino acid sequences in capsid proteins has shown selective targeting of viral vectors to cell surface receptors *in vitro*. However, these highly specific but relatively minor modifications to the capsid proteins are unlikely to address the plethora of unwanted interactions with antibodies, clotting factors and macrophages that limit non-local use of viral vectors. Theoretically, it would be possible to combine several modifications to improve vector survival and targeting, but extensive changes to capsid proteins are likely to have a negative impact on production.

9. Bi-functional antibody retargeting

Antibodies have a long history in delivering therapeutic payloads [50] and have been shown as useful targeting components for gene therapy vectors. In the context of adenoviral delivery, bi-functional antibodies are typically used that can act as a bridge between capsid proteins and cell surface receptors. Examples of successful retargeting with this approach have been demonstrated for the folate receptor [51], EpCAM [52], endoglin (CD 105) [53] and PSMA [54].

The main benefit of this strategy is the ability to retarget viruses without compromising vector integrity or existing production and purification protocols. However, it is a multi-component system that requires the manufacture of two complex biologicals: a virus and a separate antibody. Like genetic modification, it also has limited effects on vector neutralisation and clearance mechanisms.

10. Polymer coating strategies

The plethora of host–vector interactions that alter or neutralise virus particles *in vivo* has lead some researchers to consider physically separating virus particles from the environment. Polymer coating approaches are attractive for systemic delivery

where Kupffer cell clearance [55] and blood cell binding [4] are amongst many interactions that dominate tropism and distribution of virus particles *in vivo*.

Coating strategies have been mainly focused around hydrophilic polymers such as polyethylene glycol (PEG) and poly-[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA), covalently attached to the surface of virus particles [56,57]. More recently, alternative strategies have been described including the use of sugars [58].

10.1 PEG-coated vectors

PEG is a water soluble polymer [59], which has been routinely used as a drug delivery carrier in many fields. Applicability for virus delivery has been inferred from previous studies with pegylated liposomes [60] that show extended pharmacokinetics *in vivo*. In principle, a dense layer of PEG chains around the surface of a particle should resist non-specific capture by phagocytes and resist interactions with large molecules such as antibodies and complement. Several publications report the successful use of PEG for adenovirus using a variety of retargeting ligands including Herceptin [61], basic fibroblast growth factor (FGF-2) [62] and cyclic RGD [63,64]. Others have shown that PEG alone, without targeting ligands, can mediate cell entry into some cells, presumably through the amphipathic nature of PEG, causing membrane fusion [56].

However, some reports using PEG show conflicting results regarding the degree of detargeting *in vivo* [65,66], which may be due to subtle differences including the degree of modification. One important aspect of coating is the choice of chemistries for attachment of polymers to virus particles. Typically amino reactive chemistries such as succinimidyl succinate PEG (SS-MPEG) are used in most applications [67]. Alternatively tresyl monomethoxy PEG (TMPEG) has been reported to retain more activity of the virus particle [68]. The most sophisticated approach to pegylation has been developed by Kreppel *et al.* [69], whereby environmentally sensitive disulfide bonds were used to allow shedding of the polymer inside endosomes. Polymer conjugation is mediated by inclusion of cysteine residues, which undergo reduction in selective locations of the virus capsid. This combination of genetic modification and chemical retargeting provides much more definition and utility than has been achieved previously.

10.2 pHPMA-coated vectors

Poly-[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA) is a hydrophilic polymer which was first developed as a drug carrier for anticancer drugs including: farmorubicin, doxorubicin, paclitaxel and cis-platin. The addition of amino-reactive 4-nitrophenoxy groups on diglycyl side chains results in pHPMA activation [70,71] to generate a multivalent polymer. The multiple reactive groups appended to each polymer chain can react cooperatively with target lysine residues. This results in a high density of lateral modification relative to polymers that use a single point of attachment, resulting in greater steric coverage closer to the surface of the virus. *In vivo*, pHPMA coated vectors are almost completely detargeted and show extended circulation kinetics ($t_{½}$ ~ 30 min) following systemic administration [72]. In contrast, unmodified adenovirus circulates with a plasma $t_{½}$ of less than 2 min [30,35]. The benefit of pHPMA is that following coating of the virus, remaining reactive groups can be used to attach targeting moieties including growth factors, peptides and antibodies [57,73-75].

10.3 Sugars

Adenovirus is not naturally glycosylated apart from a single *O*-linked *N*-acetylglucosamine (GlcNac) located on the fibre [76]. Within the bloodstream, adenovirus stands out amongst the glycosylated proteins of the plasma and blood cell envelopes. Artificially covering adenovirus in appropriate sugar structures may help to hide the virus from immune clearance and mediate selective binding to target cells. The use of sugars for gene delivery has previously been reported within the concept of non-viral particles [77,78]. Their use in viral gene delivery is now emerging as a new approach for redirecting adenovirus from its conventional routes of cellular infection. Pearce *et al.* recently reported retargeting with chemically glycosylated adenoviral vectors. A luciferase-expressing adenovirus was shown to be devoid of natural tropism after being glycosylated with galactose and mannose but capable of selective transduction of macrophages through the mannose receptor [58]. Virus particles modified with more complex sugar structures are likely to follow in parallel with new advances in glycochemistry.

11. Pharmacological interventions and dose scheduling

A number of pharmacological interventions capable of modulating the kinetics of virus particles and other drugs *in vivo* have been described. One of the most effective agents for prolonging the circulation of adenovirus vectors is dichloromethylene-bisphosphonate-containing liposomes (Clodronate) [79]. These can temporarily interfere with Kupffer cells' function, preventing particle scavenging, the primary mechanism for vector clearance. Secondary clearance to the liver mediated through hepatocytes can be abolished by the addition of Warfarin to interfere with FX mediated infection [80]. Hence, combining both these pharmacological agents might have a potent synergistic effect.

Furthermore, the importance of dose scheduling should not be overlooked, as small predoses of adenoviral particles can be used to extend the circulation and activity of a subsequent dose [81]. Clinically, dose fractionation has been used to substantially increase the maximum tolerated dose of Newcastle Disease virus [82]. These findings may also be true for adenovirus.

Once extended circulation of viral vectors has been achieved, strategies to overcome the endothelial barrier and enhance

tumour uptake should then be considered. Drugs that increase tumour accumulation by the enhanced permeability retention (EPR) effect could become useful. Both TNF-α and bradykinin have been shown to increase vasculature permeability, resulting in improved uptake of therapeutic molecules including genes into tumour cells [83-88]. IL-2 (IL-2) is currently approved for the treatment of metastatic renal cell carcinoma and is associated with vascular permeability changes [89,90]. This property has previously been explored with macromolecules [91] and more recently with chemotherapy of soft tissue sarcoma in melphalan-treated isolated limb perfusion [92]. A similar synergistic effect with melphalan in the ILP setting has also been explored with histamine and reported to be linked with its vasoactive effects [93].

In addition to vascular permeability modulation induced by the above-mentioned agents, other pharmacological interventions to increase macromolecules uptake into tumours are possible and include improving blood flow to tumours either by restoring the transvascular pathway with agents such as: adrenaline, pentoxiphyleine or by haemodilution [94-96], or by modulating the interstitial pathway with drugs like dexamethasone, taxol and the antivascular agent 5,6-dimethylxanthenone-4 acetic acid or by radiation [97-100]. Careful use of these drugs, in combination with gene delivery vectors, has the potential to provide significant improvements in clinical outcome.

12. Expert opinion

The development of targeting strategies for therapeutic viruses has been led by laboratory and clinical observations of poor infection frequency of target cells. Initial attempts at 'retargeting' viral vectors were carried out with little attention to 'detargeting' and did not perform particularly well *in vivo*. In many ways, the early virus retargeting studies were approached with a similar mindset to antibody targeting, whereby discrete modification to receptor binding sequences can have a dramatic effect on biodistribution. However, unlike antibody molecules, virus particles are cleared rapidly from the circulation by multiple mechanisms, not least Kupffer cells that remove particles seemingly regardless of any capsid protein modification.

For the ultimate goal of gene delivery in humans, detargeting must encompass all interactions that can neutralise or sequester virus particles in a way that prevents them reaching target cells. Of the strategies published so far, only coating strategies can have a chance of tackling multiple unwanted interactions by physically separating the virus, at least temporarily, from its environment. However, it is likely that a combination of strategies will be required to achieve systemic efficacy in humans, including the use of chimeric or genetically modified viruses delivered within a polymer coat.

This task of effective gene delivery is made particularly challenging by the lack of suitable laboratory models that are able to predict virus behaviour in humans. For example, adenovirus type 5 is found associated to erythrocytes in human blood, but no significant erythrocyte association can be found in the blood of mice. This fundamental difference questions the relevance of any murine i.v. study involving HAdV-5, unless of course the vector has been modified in such a way that erythrocyte binding is no longer an issue in either case.

The use of more stringent and assay systems that closely represent the clinical environment will help to make further progress for the field of gene delivery. Relatively simple studies involving human blood or blood components will provide much more information about vector survival than comparative studies in mice.

Should extended plasma circulation be achieved, retargeting must be approached with an understanding of the physical limitations of virus particles such as the constraints on diffusion, extravasation and tissue penetration. Targeting the tumour-associated vasculature is one strategy that may prove beneficial for adenovirus, avoiding the need for virus particles to escape from the bloodstream.

The seemingly insurmountable challenge of delivering adenovirus in humans had led most clinical trials to adopt local administration protocols. Direct administration of virus particles to tumours, or regions containing tumours, provide a way to demonstrate mechanism of action in the clinics. However, limiting the use of versatile gene therapies to superficial tumours is very unsatisfying for most in the field and there is an irresistible pull towards the ultimate goal of effective systemic delivery.

Declaration of interest

Kerry Fisher holds equity in Hybrid Systems Ltd.

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Affiliation

Houria Bachtarzi MPharm (Hons),
Mark Stevenson PhD & Kerry Fisher PhD†
†Author for correspondence
University of Oxford,
Department of Clinical Pharmacology,
Old Road Campus Research Building,
OX3 7DQ, Oxford, UK
Tel: +44 0 1865617043; Fax: +44 0 1865617100;
E-mail: Kerry.Fisher@clinpharm.ox.ac.uk