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REFLECTION PAPER ON *IN VITRO* INVESTIGATION OF MITOCHONDRIAL TOXICITY OF ANTI-HIV NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

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1. INTRODUCTION

Mitochondrial dysfunction is a major toxicity of Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs/NtRTIs¹). NRTIs/NtRTIs are part of the highly active antiretroviral therapy (HAART) of HIV infections. The fact that mitochondrial toxicity of NRTIs is strongly suggested to be responsible for many adverse effects observed in patients under HAART has led to increasing regulatory requests for *in vitro* investigation into mitochondrial toxicity of NRTIs and NRTI combinations. However, no regulatory standards for using established models are available at present.

The aim of this reflection paper is to review recent experiments with *in vitro* investigations of mitochondrial toxicity of NRTIs in cell culture models and provide some principles to consider for the conduct of such studies in order to ensure more consistency in testing.

2. BACKGROUND

2.1 Clinical impact of NRTI-induced mitochondrial toxicity and importance of *in vitro* testing

During the last twenty years major progress has been made in the understanding and treatment of human immunodeficiency virus (HIV) infection. Different classes of antiretroviral agents have been authorised for the treatment of HIV infection, such as nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion inhibitors (FIs).

Current treatment guidelines recommend the use of combinations of multiple antiretroviral agents in HIV therapy which usually contains one or more NRTI. This therapy known as HAART (Highly Active Antiretroviral Therapy) is now the standard HIV therapy and has led to a substantial reduction in morbidity and near complete suppression of HIV replication. But since HAART is unlikely to eradicate HIV, lifelong antiviral therapy may be required. This is the reason why increasing attention is paid to the long-term safety of HAART.

A wide range of adverse effects associated with mitochondrial toxicity has been recognised in HIV infected patients after medium-term to long-term treatment with NRTI-bound therapies [Brinkman et al., 1998; Moyle, 2000a]. The main adverse effects include lactic acidosis, hepatic steatosis, neuropathy, (cardio-) myopathy, pancreatitis, and probably lipodystrophy [Carr and Cooper, 2000; Squires, 2001; Dagan et al., 2002]. Mitochondrial toxicity could pose a major threat to successful long-term HIV therapies and is of great concern for children exposed in-utero and/or postnatally to NRTIs [Blanche et al., 1999; Haas, 2000], especially if HIV-uninfected. The long-term consequences for these children are currently not known. Furthermore, combination regimes may induce additive or synergistic mitochondrial toxic effects or may lead to the development of new tissue targets.

The fact that mitochondrial toxicity of NRTIs is strongly suggested to be responsible for many adverse effects observed in patients under HAART has led to increasing regulatory requests for *in vitro* investigation into mitochondrial toxicity. The study designs of the cell culture models currently used in the investigation of NRTI-induced mitochondrial toxicity varies greatly making it difficult to compare findings from different applicants, i.e. it is difficult to distinguish protocol-specific differences from compound-specific ones.

Recommendations are needed to obtain uniform study designs in the *in vitro* testing of mitochondrial toxicity of NRTIs. Uniformity would improve the comparability of findings presented by different applicants and would allow for a rank order of potencies of the compounds/combinations tested.

This paper is an attempt to discuss the relevance of the *in vitro* studies conducted so far for the investigation of NRTI-induced mitochondrial toxicity and to give some proposals to the *in vitro*

¹ Abbreviated to NRTIs

evaluation of mitochondrial toxicity of anti-HIV therapeutics. This paper applies mainly to new NRTIs and new combinations of NRTIs intended to be used clinically and may also be applied to NRTIs or NRTI combinations already marketed where appropriate (e.g. postmarketing observation of adverse effects).

The proposals given in this paper may also be applied to new active substances for human use when there is concern about potential mitochondrial toxic effects.

2.2 Mechanism of mitochondrial toxicity

Currently approved NRTIs include structural analogues of deoxyadenosine, guanosine, cytidine, or thymidine, as well as an analogue of deoxyadenosine monophosphate, which can serve as substrates for viral reverse transcriptase (RT). These agents suppress viral replication after phosphorylation by intracellular kinases to the active triphosphate (TP) form and by incorporation into viral DNA. Due to chemical modifications to the 3'-OH group of the deoxyribose sugar, NRTIs prevent the addition of the next nucleotide, leading to premature termination of the growing viral DNA strand during reverse transcription [Kakuda, 2000; Squires, 2001; Anderson et al., 2004]. NRTI-TPs can also be substrates for cellular DNA polymerases (pol). The general inhibitory effect of NRTIs on these polymerases relative to HIV-RT is as follows: HIV-RT \gg DNA pol γ > DNA pol β > DNA pol α = DNA pol ϵ [Lewis et al., 2003]. The highest affinity is to DNA pol γ , the only enzyme responsible for the replication of mitochondrial (mt) DNA. NRTIs compete with endogenous nucleotide triphosphates for incorporation into growing DNA chain by DNA pol γ . The terminally incorporated NRTI halted replication of mtDNA and persists in DNA due to resistant to exonucleolytic removal by the exonuclease activity of DNA pol γ [Lewis et al., 2003; Lee et al., 2003].

Based on this mechanism, the "DNA pol γ hypothesis" was developed [Lewis and Dalakas, 1995]. Besides DNA pol γ inhibition, this hypothesis also suggests that the tissue and substance specific features of NRTI-induced mitochondrial toxicity in patients may be due to (i) tissue concentration of the substance, (ii) the level of substance activation by cellular nucleoside kinases to the respective triphosphate (iii) the ability of the triphosphate to inhibit DNA pol γ either competitive or non-competitive and (iiii) dependency of a given tissue upon mitochondrial function. Later on this hypothesis was amended to add oxidative stress and mtDNA mutations in the "mitochondrial dysfunction hypothesis" [Lewis et al., 2001; Lewis et al., 2003].

Also other mechanisms, either secondary to or independent of inhibition of DNA pol γ , are assumed to be involved in NRTI toxicity [Moyle, 2000b; Lewis et al., 2003; Lund and Fallace, 2004, Cote, 2005].

3. COMPARISON OF EXISTING IN VITRO MODELS FOR THE INVESTIGATION OF NRTI-INDUCED MITOCHONDRIAL TOXICITY

Mitochondrial toxicity of NRTIs has been investigated in several *in vitro* studies, such as enzyme kinetic studies and cell culture studies.

Enzyme kinetic studies

The determination of the kinetics of incorporation and exonuclease removal of NRTI-TPs catalyzed by isolated DNA pol γ is a simple and rapid *in vitro* method to screen agents for their mitochondrial toxicity potential [Lee et al., 2003; Johnson et al., 2001].

In vitro inhibitory activity towards mtDNA synthesis by DNA pol γ is a first sign of an agent's potential to induce mitochondrial toxicity [Lee et al., 2003]. In this way those agents can be identified which exhibit mitochondrial toxicity through inhibition of DNA pol γ or incorporation into mtDNA by DNA pol γ . However, lack of effect on DNA pol γ does not preclude mitochondrial toxicity since there are many other contributing factors such as effects on mitochondrial enzyme function or oxidative stress. Therefore, enzymatic kinetic studies provide limited predictive evidence of clinical toxicity. Those assays might be useful to identify compounds with a high potential for toxicity early on during development of new active substances.

Cell culture studies

Cell culture studies are able to investigate effects on mtDNA as well as effects on mitochondrial function. In contrast to studies in isolated DNA pol γ enzyme, they are also useful to reveal effects on mitochondrial function not associated with the inhibition of DNA pol γ .

A range of different cell culture models have been used so far. The sensitivity of cells to detect potential mitochondrial toxicity of NRTIs varies between different cell types possibly because of differences in their capability to activate the NRTIs to their triphosphate form, depending on the activation state of the cell [Kakuda, 2000; Anderson et al., 2004; Rylova et al., 2005]. For example, stavudine (d4T) and zidovudine (ZDV) are more active in activated (proliferating) cells and other NRTIs are more active in resting cells.

Initial cell culture studies with NRTIs examined mitochondrial toxicity in T-lymphoblastoid cells [Chen et al., 1991; Medina et al., 1994; Martin et al., 1994]. They are rapidly growing and poorly differentiated cells and energy production rather depends on glycolysis than on oxidative phosphorylation. Data obtained from these cells seem to be not fully predictive of clinical toxicity [Höschele, 2006]. The studies in T-lymphoblastoid cells demonstrated that zalcitabine (ddC) and d4T are the most potent inhibitors of mtDNA synthesis followed by didanosine (ddI) and ZDV. This potential does not correlate with the potential to inhibit DNA pol γ by the corresponding triphosphate forms ($ddC-TP > ddA-TP > d4T-TP \gg 3TC-TP \geq PMPApp \geq ZDV-TP \geq CBV-TP$). For example, in T-lymphoblastoid cells d4T causes more pronounced mtDNA depletion than ddI, although ddA-TP (active form of ddI) is a more potent inhibitor of DNA pol γ . This could be explained by differences in anabolism of individual compounds in different cell cultures. ZDV seems to have a higher clinical toxicity than would be predicted on the basis of studies in T-lymphoblastoid cells. The potential of ZDV to induce depletion of mtDNA content in CEM- and MOLT-4 cells or to increase medium lactate levels in CEM-cells was low, compared to ddC and d4T. Although these effects seem to correlate with the low potential of ZDV-TP to inhibit DNA pol γ , in patients treated with ZDV myopathy and cardiomyopathy associated with mitochondrial abnormalities were observed [Dagan et al., 2002]. These adverse effects were suggested to result from direct effects of ZDV on mitochondrial function rather than on inhibitory effects of DNA pol γ [Lewis et al., 2003; Lund and Fallace, 2004].

Cell culture models using T-lymphoblastoid cells might be more sensitive for those agents that are preferentially activated in highly proliferating tissues and could be used for the prediction of mitochondrial toxicity of similar high turnover populations *in vivo*. However, as in patients mitochondrial toxicity becomes primarily manifested in resting or slowly dividing tissues like muscle, liver, brain, pancreas, and adipocytes, data obtained from T-lymphoblastoid cells seem to be not fully predictive of clinical toxicity for the whole range of NRTIs [Höschele, 2006].

The tissues mainly affected by mitochondrial toxicity of NRTIs showed a high metabolic activity and as such are highly dependent on mitochondrial function. In several cell culture models the mitochondrial toxicity of NRTIs was investigated in cells originating mainly from the mentioned tissues, i.e. human hepatoblastoma cells (HepG2), primary human skeletal muscle cells, human renal proximal tubule cells differentiated rat pheochromocytoma cells (PC-12), and human pancreatic cells (Capan-1) [Birkus et al., 2002; Pan-Zhou et al., 2000; Walker et al., 2002; Benbrik et al., 1997; Cui et al., 1997; Lake-Bakaar et al., 2001]. The main emphasis of these studies was to examine the potential of different NRTIs to induce mitochondrial effects in different cells, in order to obtain insight into organ-specific toxicities of individual NRTIs in patients.

A comparison of the different tissue-specific cell cultures currently used for the investigation of NRTI-induced mitochondrial toxicity revealed no marked differences in the potential of various NRTIs to inhibit mtDNA replication or affect mitochondrial function [Höschele, 2006]. The degree to deplete mtDNA by NRTIs showed an overall rank-order of $ddC > ddI > d4T \gg ZDV \geq 3TC = ABC = TDF$, which correlates with the potential of these agents to inhibit DNA pol γ . The studies in different cell culture models are not marked enough to recognise tissue-specific mitochondrial effects of individual NRTIs or to obtain explanations for their organ-specific toxicities in HIV-infected patients. Cell culture studies should be regarded as more useful to investigate the potential of an agent to induce

mitochondrial toxicity. An advantage of cell cultures over studies in isolated DNA pol γ enzyme is that cell cultures are also useful to reveal effects on mitochondrial function not associated with inhibition of DNA pol γ . Therefore, studies in cell cultures seem to be more valuable for the investigation of NRTI-induced mitochondrial toxicity *in vitro* in early preclinical development.

Based on these general considerations in the next section proposals for a study design relevant for the investigation of NRTI-induced mitochondrial toxicity is given.

4. REFLECTION ON A STUDY DESIGN FOR THE INVESTIGATION OF NRTI-INDUCED MITOCHONDRIAL TOXICITY

The purpose of *in vitro* testing for mitochondrial toxicity of anti-HIV agents is eventually aimed at identifying those substances which might have the potential to induce mitochondrial toxic effects in patients. The use of cell cultures is recommended as they, in contrast to studies with isolated enzymes, provide the possibility to detect effects on mtDNA as well as on mitochondrial function.

It is to be preferred that the cell line selected is well-established in the investigation of NRTI-induced mitochondrial toxicity. The stability of the cell line should allow for long-term treatment. The cell line should be able to activate NRTIs when tested as single compound or in combination. It is important that the cell line contains a large number of mitochondria and mtDNA and is able to clearly show effects on mtDNA and on mitochondrial function.

At present the human hepatoblastoma cell line (HepG2) is considered to be a suitable model for the investigation of NRTI-induced mitochondrial toxicity [Pinti et al., 2003;Höschele, 2006; Lund et al., 2007]. This cell line fulfils the above mentioned requirements since it has large amounts of mitochondria and mtDNA and is highly sensitive for recognition of effects on mtDNA and mitochondrial function and seems to be a promising candidate for the investigation of NRTI-induced mitochondrial toxicity. The use of further cell types (e.g. cells of lymphocytic origin like CEM) could be considered in case were the investigation in HepG2 cells leads to ambiguous results.

Preferably, mitochondrial toxicity should be investigated in extended study periods of up to 2 to 3 weeks over several passages. However, too long study periods leading to unhealthy culture conditions or to the onset of substance-resistant clones should be avoided because these could confound the interpretation of data (Ferraresi et al, 2004).

Medium and test substance should be replaced as appropriate. Sampling should be performed during the course of the study at defined time intervals. Time intervals of 3 to 5 days have been shown to be appropriate [Walker et al., 2002]

Each substance should be investigated over a wide range of concentrations with at least 3 different concentrations. In the early phase of preclinical development the range of concentrations should be orientated to in-vitro data on pharmacodynamic (e.g. EC₅₀ for virus inhibition) and toxicity (e.g. TC₅₀). Concentrations in the range of EC₅₀ and multiples should be investigated. However, cytotoxic effects should be avoided.

The endpoints should have been well established for the purpose of investigation of NRTI-induced mitochondrial toxicity in cell cultures and should be measured by using scientifically valid methods.

The following endpoints are considered as standard (i) quantification of mtDNA content (ii) measurement of lactate release into medium and (iii) cell number and cell viability.

The measurement of the mtDNA content has been reported to be the most sensitive indicator of NRTI-induced mitochondrial toxicity compared to other endpoints (cell viability, mitochondrial morphology) [Medina et al., 1994]. This endpoint has been used in a wide range of *in vitro* cell culture studies investigating NRTI-induced mitochondrial toxicity.

A further widely used marker of NRTI-induced mitochondrial toxicity is the measurement of lactate in the medium of cell cultures marker.

Lactate is the endpoint of oxygen-independent cellular energy production. If mitochondrial function is affected following treatment with NRTIs, NADH formation from glycolysis by oxidation of pyruvate via the citric acid cycle may be impaired. This results in a shift towards anaerobic oxidation of pyruvate by lactate dehydrogenase and the formation of lactate. Accordingly, elevated lactate levels are a sign of mitochondrial dysfunction.

Cell viability is not a direct measure of mitochondrial toxicity but it is commonly used to indicate cytostatic and/or cytotoxic events/effects. Such events/effects might lead to difficulties in the interpretation of mitochondrial toxic effects.

Additional endpoints (supportive endpoints) for the detection of mitochondrial toxicity could be used, such as assays of mitochondrial function (e.g. ATP content, enzyme activities), measurement of the mitochondrial membrane potential, mitochondrial RNA levels or quantification of mitochondrial proteins, activity of cytochrome C oxidase, measurement of mitochondrial mass and analysis of mitochondrial morphology. However, in the interest of a clear interpretation of the results, well-established endpoints should be used in investigations of mitochondrial toxicity in cell cultures.

Inclusion of a positive control is strongly recommended in order to ensure the functionality of assays and methods and the general suitability of the cell culture model. ddC is considered as appropriate agent for positive control, since its effects on mitochondria are well characterized.

ABBREVIATIONS

3TC	Lamivudine
ABC	Abacavir
ATP	Adenosine Triphosphate
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
HAART	Highly Active Antiretroviral Therapy
HepG2	Human Hepatoblastoma Cells
HIV	Human Immunodeficiency Virus
mtDNA	Mitochondrial Deoxyribonucleic Acid
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
Pol	polymerase
RT	Reverse Transcriptase
TDF	Tenofovir Disoproxil Fumarate
TP	Triphosphate
TP	Triphosphate
ZDV	Zidovudine

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