

RESPONSE TO DR. DAVID BACK BY THE PERTH GROUP

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1. In vitro studies of the IC₅₀ for HIV reverse transcriptase

Most of Back's effort in this section is to convince us that "data generated in vitro must be extrapolated with caution to the in vivo situation". In our paper we were even more emphatic. On page S3 we wrote: "In vitro data cannot be extrapolated in vivo". In fact one of our criticisms in our paper is the introduction of AZT into clinical practice solely on the basis of two in vitro studies.

He claims that IC₅₀ is totally dependant on the experimental condition such as dTTP concentration, the template-primer used, the cell line used and the stage of the cell cycle. This later condition is also stressed by the authors of one of Back's

references (Fletcher et al). They stated: "... (zidovudine and stavudine) are preferentially phosphorylated in activated cells and yield higher ratios of intracellular triphosphate to endogenous deoxynucleoside triphosphate than in resting cells". Again, this is something which we have stressed repeatedly. According to Back "The concentration of AZTTP and dTTP in vivo is difficult to measure", and that there is "variability of dTTP levels between and within patients". This means that it is not possible to compare Furman's dTTP concentration with the in vivo concentration.

Because:

1. Furman used the synthetic template-primer An.dT15. This template primer is transcribed not only by the viral RT but also by the cellular DNA polymerase β and γ . This means, inhibition of these enzymes will be interpreted as a lower IC_{50} for HIV RT;
2. Furman used "purified HIV reverse transcriptase". (It will be easier to inhibit a purified enzyme than one in the complicated cellular environment);
3. Furman used mitogenically stimulated cells (in AIDS patients the cells are dormant. In fact many, if not all of them, will be abnormal, if not dead);

We concluded that Furman's conditions were ideal for obtaining a low IC_{50} and a high triphosphorylation of AZT. If Furman's IC_{50} was $0.5\mu\text{M}$ then we would expect in vivo to be higher. In other words the meaning of our "ideal" is opposite to that of Back's ("The only ideal condition will be those that reflect as closely as possible the in vivo situation").

Also in this section for some unknown reason Back introduced the term K_i that is the "inhibition constant of a drug for enzyme". Why introduce K_i when IC_{50} is the concentration of AZT necessary to inhibit the RT activity by 50% or produce a 50% decline in virus production, the latter ultimately being the only clinically relevant parameter? Interestingly, when Back attempted to prove that AZT has antiviral activity (see below) he used IC_{50} not K_i . Back claims: "That AZT has antiviral activity in vitro is undeniable. My research group has recently demonstrated that in persistently infected U-937 cells (a human T-cell derived immortal cell line) the IC_{50} for AZT (that is the concentration of AZT required to give a 50% drop in virus production) was 0.05 μ M (Hoggard et al 2000)"

However:

1. The IC_{50} for antiviral activity was defined as the concentration which "decreases p24 antigen production by 50%";
2. Under "Acknowledgments" Back et al wrote: "We are grateful to the MRC and BSAC for financial support. We are also grateful to Dr G Reid, Professor L Montagnier and the NIBSC Centralised Facility for AIDS Reagents for donation of antiserum to HIV-1 p24 and HIV infected U937 cells";
3. In 1997 Montagnier admitted that p24 which in 1983 he claimed to be HIV (a claim accepted by all HIV experts, including Back), actually originated from material which did not contain even retrovirus particles, much less

HIV. This is as good an evidence as it can be that p24 is a cellular protein.

In other words, in 2000 Back proved that AZT is toxic.

4. **IN VIVO PHOSPHORYLATION OF AZT**

“5.1 As indicated above, AZT is only effective in inhibiting reverse transcriptase (and hence viral replication ARB PLEASE CHECK IF THE BIT HERE IN BRACKETS IS BACK’S OR OURS? IF IT’S OURS IT HAS TO BE IN SQUARE BRACETS) in its triphosphorylated form. The Plaintiffs allege (in paragraph 16.2 of the Particulars of Claim) that "AZT is triphosphorylated insignificantly in vivo" and that the best designed and executed studies indicate that AZT is "triphosphorylated in vivo to levels one or more orders of magnitude below the drug's IC₅₀ value, as determined by Furman et al in ideal in vitro conditions". I have already dealt with the IC₅₀ data of Furman et al above and demonstrated that: (1) IC₅₀ values depend on the experimental conditions in which they were determined and accordingly vary greatly from laboratory to laboratory; (2) there is no basis for the statement that Furman's conditions were "ideal"; and (3) it is wholly inappropriate to extrapolate from an in vitro measurement of IC₅₀ to the in vivo situation. I will now address the in vivo data on AZT phosphorylation".

Since:

- I. “...AZT is only effective in inhibiting reverse transcriptase in its triphosphorylated form”;

II. In our paper we have shown that all the presently available data show that AZT has no effect on the viral load, Back has not presented any evidence of his own or of anybody else to show that AZT decreases the viral load;

It means that “AZT is triphosphorylated insignificantly *in vivo*”. In other words, Back appears to have missed this important point.

“ 5.2 In this context, the use of the word "insignificantly" by the Plaintiffs is not appropriate since numerous studies by different investigators (please see Annex 2) have clearly demonstrated that intracellular concentrations of AZTTP greater than the *in vitro* K_i value can be determined in peripheral blood mononuclear cells (PBMCs) from HIV positive patients receiving zidovudine-containing therapy. Furthermore it is important to remember that the "significance" of the antiviral effect of the drug (which occurs only via triphosphorylation) has been established in the numerous clinical trials that have shown that AZT alone or as part of a combination of other drugs is extremely beneficial to HIV patients".

In Hayman's particular of claim no mention is made of K_i . Since IC_{50} is the most relevant parameter, it is the only one discussed. Back does not give any evidence that the *in vivo* phosphorylation of AZT is "significant", that is, it will be sufficient to "produce 50% decline in virus production". Neither does he give any evidence in support of his claim

"that AZT alone....is extremely beneficial to HIV patients". All the studies, including the Concord show the opposite.

"5.3 My research group has been at the forefront of developing methodologies for intracellular anabolite determinations in vivo (including Barry et al, 1994; Barry et al, 1996; Phiboonbanakit et al, 1996; Wattanagoon et al, 2000; Moore et al, 2000; Hoggard et al, 2001; Kewn et al, 2002). It is important to observe that the different analytical methodologies used both by my group and others (see Annex 2) give comparable measurements of intracellular AZTTP.

Examples of the different methodologies used are (1) high performance liquid chromatography - radioimmunoassay; (2) solid phase extraction - tandem mass spectrometry; (3) cartridge - radioimmunoassay; (4) enzymatic - primer extension. As in all areas of clinical science, methods have been refined and levels of assay sensitivity improved as technology and know-how has advanced. However, in vivo studies annexed to the Particulars of Claim are some of the pivotal clinical studies in this area. More recently there have been advances in methodologies such that we now have assays with greater sensitivity with a consequent lowering of the limit of quantitation".

In this paragraph no evidence is present in support of AZT as an antiretroviral or of any benefits to the patients by any means. It is laudable that David Back and his team have put great effort in developing methods for measuring triphosphorylated AZT and endogenous nucleotides. However, the fact that so many methods exist suggests that none of them is satisfactory. Even if they are all

perfect, it does not help us answering the only question which all of us and especially the patients are interested: does AZT have an antiretroviral effect?

"5.4 In clinical studies, the majority of values of AZTTP concentration as calculated by the various methods listed above, lie between 0.04 and 0.15 pmoles/ 10^6 cells (values will depend amongst other things on the timing of the sample). Based on the volume of a single PBMC these values can be expressed as "micromolar" concentrations. The average PBMC volume is 0.4 picolitres (data based on FACS analysis or Coulter counter analysis). Therefore the intracellular AZTTP concentration is in the range 0.1 - 0.36 μ M, i.e. about ten-fold greater than the computed K_i values, listed above, of 0.01 - 0.04 μ M (see paragraph 4.10). So if we are to attempt any in vitro - in vivo correlation (and we have to bear in mind all the caveats previously listed), it points to the presence of inhibitory concentrations of AZTTP in vivo and not to "insignificant" phosphorylation".

David Back, by introducing K_i again, just muddies the waters.

Where is the evidence that in vivo AZT is significantly phosphorylated to "produce 50% inhibition of enzyme activity or, 50% decline in virus production"?

"5.5 Two recent seminal papers should be highlighted.

- (A) Firstly, Fletcher et al 2000 reported on zidovudine triphosphate and lamivudine triphosphate (3TC) concentration - response relationships in HIV-infected persons. They concluded that two commonly used markers of HIV infection, the percent change in

CD4 cells during therapy (CD4 count) and the rate of decline in HIV RNA (viral load) in plasma were related to the intracellular concentrations of zidovudine and lamivudine triphosphates, i.e. at higher levels of AZTTP and lamivudine triphosphate the increase in CD4 count and decrease in viral load is greater than when the concentration of triphosphorylated AZT and 3TC is lower. This study is important because it shows a direct correlation between AZT triphosphorylation and the immune response in HIV positive patients.

- (B) Secondly, Hoggard et al (2002) in the CHARM study have examined the intracellular phosphorylation of zidovudine, lamivudine and abacavir over 48 weeks in 22 HIV patients recruited in the Department of Medicine, Somerset Hospital, Cape Town. The novel feature of this study was that all drug triphosphates and endogenous deoxynucleoside triphosphates were assayed. This enabled calculation of the ratio of drug triphosphate to endogenous triphosphate. Since AZTTP and dTTP are "competing" for incorporation into the growing DNA strand it is the ratio between the two that is important rather than simply the absolute concentration of AZTTP. Importantly in the Hoggard et al study the level of AZTTP was found to be in the range 0.02 - 0.2 pmoles/ 10^6 cells and the ratio of AZTTP:dTTP was shown not to change over 48 weeks, indicating that there were no potentially adverse time dependent changes in the phosphorylation profiles i.e. the ratio of drug triphosphate to endogenous triphosphate did not

decrease over the course of the study. This study represents the most comprehensive data-set available for the study of AZTTP levels (i.e. 22 patients with data at week 0, 2, 6, 12, 24 and 48). It represents a total of more than 250 drug triphosphate determinations with an equivalent number of dTTP determinations".

The Fletcher et al study: (i) was not blind and there was no placebo; (ii) there is no evidence for the validity of the method used to measure AZT-TP; (iii) they reported an inverse relationship between AZP-TP and CD4. This is not biologically plausible, and, as they admit contrary to the findings of other researchers; (iv) it is not possible to know what effect the combination of the drugs, much less each drug had on the absolute changes in the viral load and CD4 cell counts. (Fletcher et al gave only % changes).

To claim that "this study is important because it shows a direct correlation between AZT triphosphorylation and immune response in HIV positive patients", is to go beyond the evidence.

According to Hoggard et al: "The CHARM study is a large multi-centre trial evaluating the effect of antiretroviral nucleotide analogue combination therapy in 229 patients". However, the only findings presented in the Hoggard et al paper is changes in phosphorylation over time in endogenous deoxinucleotide

triphosphate (dNTP), abacavir, lamivudine and AZT in 22 patients, in the presence of hydroxyurea.

Back's claim that the level of AZTTP was 0.02 - 0.2 and that "This study represents the most comprehensive data-set available for the study of AZTTP levels can be questioned on several grounds, including the following:

- (i) Since: the patients were simultaneously exposed to another two nucleosides analogues; and since they all compete for reducing equivalent which is necessary for triphosphorylation; in this situation one will expect a lower level of AZTTP not higher than that reported in other studies;
- (ii) There was extreme variability of AZTTP between individuals. "There were also median sixfold, 13-fold and 11-fold differences in CBVTP, ZDVTP {AZTTP} and 3TCTP concentration respectively between the highest and lowest values within each patient over the time course of the study".
- (iii) There is no evidence that the level of AZTTP reported by Back has any antiviral effect or immunological and clinical benefits.

In the Hoggard, Back et al study, "viral loads and CD4 cell counts were monitored to 72 weeks". However, for some unknown reason no data is given regarding the CD4 cells. Regarding the viral load they stated: "In the first 24 weeks of study, five patients developed virological failure". No mention is made as to what happened to week 72;

- (iv) The fact that the treatment protocol used by Back and his associates (3 nucleoside analogues + hydroxyurea) is not used in clinical practice, suggests that the Cape Town study was an unsuccessful experimental exercise.

“5.6 The Plaintiffs have placed too much emphasis on the absolute concentration of AZTTP without considering also the concentration of dTTP with which it is competing. Since it is the ratio of AZTTP:dTTP that will ultimately determine antiviral response, it is important that data are generated for both anabolites; this gives important additional information. If, for example, AZTTP levels were reduced in a patient but the dTTP levels were also low, then the competing ratio would not necessarily be different from a patient with higher AZTTP and, also, higher dTTP. Enzyme inhibition would likely be comparable. So, if dTTP is low in cells, then less AZTTP will be required to inhibit reverse transcriptase. The recent advances in methodologies to measure both components should enable dose-response (pharmacokinetic-pharmacodynamic) relationships between phosphorylation and clinical effect to emerge”.

We agree, "if dTTP is low in cells, then less AZTTP will be required to inhibit reverse transcriptase". In fact if the dTTP is low the enzyme will be inhibited even if AZTTP is zero. However, in this situation all the other DNA polymerases will also be inhibited, resulting in cellular and patients death or at best, abnormal cells and even more sick patients

CONCLUSION

1. In our paper we presented ample evidence that AZT has no effect on viral isolation, p24 detection and viral loads, that is AZT has no antiretroviral effect. Back ignored all this evidence. Why?
2. Neither in his report nor anywhere else (including his references) does Back present any evidence which proves AZT has antiviral effects and clinical benefits. No matter how high the level of AZTTP and how fancy Back's footwork the ultimate test is whether AZT has any ARV effect. Nowhere in Back's commentary can one find even suggestive evidence for such an effect.
3. David Back did not present any evidence which contradicts paragraphs 15 and 16 of the Hayman particulars of claim.