Is Stavudine Triphosphate a Natural Metabolite of Zidovudine?

Our group has published evidence of the presence of intracellular stavudine triphosphate (d4T-TP) in patients treated with zidovudine (AZT) (1). In a recently published paper, Melendez et al. (6) present clinical data in complete contradiction with our finding, since they failed to detect d4T-TP in peripheral blood mononuclear cells (PBMCs) from more than 500 patients on AZT therapy. It is worth noting that we use a technique allowing direct determination of nucleoside reverse transcriptase inhibitor (NRTI) triphosphates, whereas Melendez et al. use an indirect method involving removal of NRTI phosphate before high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) analysis. To explain these contradictory results, Melendez et al. suggest that in our experiments d4T-TP is produced artificially during the HPLC–MS–MS process. We would like to comment on this and other points raised by Melendez et al.

Our demonstration of the presence of d4T-TP in PBMCs from patients on AZT therapy is based on a fully validated method (2) routinely used in our laboratory (7, 9, 10). We have never observed d4T-TP in any of hundreds of samples, processed in a number of clinical labs, from patients treated with any drug other than d4T or AZT. This therefore excludes the cross-contamination or interference alluded to by Melendez et al.

The hypothesis that d4T-TP is produced artificially during the HPLC–MS–MS process is untenable because the spiking of cells with AZT-TP before processing does not lead to the production of any d4T-TP.

The methodology used by our laboratory (especially ion pairing with dimethylhexylamine [DMH]) is not in question since it has been used successfully by many other groups (4, 5, 8). We note that Melendez did not succeed in using the same methodology; thus, unfortunately, a direct comparison could not be made.

Since 2003, we have confirmed our initial findings with a larger number of individuals on AZT therapy by using new methods allowing direct and simultaneous determination of d4T-TP and AZT-TP in the same sample. In addition, we have demonstrated the presence of intracellular d4T-TP by using an indirect method similar to the one used by Melendez et al. These data are included in a paper in preparation.

Finally, in their paper, Melendez et al. did not reference an independent study demonstrating the presence of d4T in plasma from patients treated with AZT (3), thus indirectly demonstrating the *in vivo* production of d4T from AZT. We have been able to confirm this observation recently (unpublished results).

In conclusion, there is clearly a complete disagreement between our data and those reported by Melendez et al., but this discrepancy cannot simply be explained by crude analytical error or contamination due to our methodology. In addition, we cannot discount the possibility that the intracellular metabolism of AZT differs between the two study populations. We feel that this discrepancy should be investigated through an exchange of *in vivo* and artificially spiked samples between our two laboratories, so that they can be analyzed by the two techniques. We are willing to participate in such a study under the supervision of an independent laboratory.

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Authors' Reply

Recently, our group at the University of Puerto Rico reported the lack of evidence for the *in vivo* transformation of intracellular zidovudine triphosphate (ZDVTP) into stavudine triphosphate (d4TTP) (9). Our findings are not consistent with results previously reported by Becher et al. (2). The arguments that support our results are fully explained in the published article (9). In response to our report, Benech et al. submitted a correspondence commenting about various points stated in

our article. Thus, we would like to respond to the comments made by Benech et al.

Our report showing the lack of evidence for the *in vivo* transformation of ZDVTP to d4TTP is based on a fully validated method used in our laboratory for several years (4, 6, 12, 13). The sensitivity and limits of detection for both ZDVTP and d4TTP are comparable to the direct methodology used by Becher et al., as established in our article (9).

It is important to clarify that not only experiments with clinical specimens but also *in vitro* experiments were analyzed, and the same results were obtained, that is, there was no evidence of intracellular ZDVTP transformation (9).

The clinical specimens were obtained from various patient populations (children and female and male human immunodeficiency virus [HIV]-infected and HIV-hepatitis C virus-coinfected adults), and none of the populations studied presented the *in vivo* transformation (9).

The ion-pairing chromatography for the separation and quantification of other nucleotides has been implemented successfully by other groups (5, 7, 10); however, the chromatographic conditions were quite different from those reported by Becher et al. In all of the three referenced studies, the mobile phase was adjusted to pH 7.0 instead of the pH 11.5 used by Becher et al. As mentioned in our article, the chemical reduction of ZDVTP to d4TTP is favorable at high pH values (11). In addition, the DMH concentrations in two of the methodologies (7, 10) were lower than 20 mM, since significant ion suppression was encountered at higher concentrations. Qian et al. used the adduct ions of DMH with AMP, ADP, and ATP as the quantitative ions, because they provided better sensitivity than the method selecting the $[\text{ATP} + \text{H}]^+$ ions (10). Fung et al. selected the mass transitions resulting from the cleavage of the respective glycoside linkages for the quantification of zidovudine and carbovir nucleotides (5). These mass transitions gave characteristic daughter ions for each analyte, instead of the common general pyrophosphate daughter ion ($m/z = 159$) used by Becher et al. (1). Kalhorn et al. also used DMH as the ion-pairing reagent for the HPLC-MS quantification of fludarabine triphosphate, along with adenosine phosphates; however, the mass spectrometry detection mode was by selected ion monitoring of the parent ion (7).

Furthermore, King et al. reported recently that they failed to replicate the Becher et al. direct HPLC-MS-MS methodology for intracellular ZDVTP determination (8). King et al. could not reproduce the Becher et al. direct methodology because of supply and methodology issues, as stated in their report (8). They observed that ion-pairing content suppressed the electrospray ionization, and, moreover, they had seen intense bleeding of the ion-pairing reagent over 1 year after their use of the system, causing carryover issues during the method development. For that reason, King et al. decided to use the indirect methodology previously validated in their laboratory. Their indirect methodology for the ZDVTP determination was not only highly specific and sensitive but also showed a strong correlation with the established immunoassay method.

In addition, King et al. discussed a concern they had with environmental contamination that led to an apparent interference during the HPLC-MS-MS method development. They found that the interference came from the environmental contamination of ZDV, since they had been working with NRTI plasma determination at the same laboratory for several years. King et al. concluded that this issue should be taken into consideration when using such highly sensitive HPLC-MS-MS methods, no matter whether the methodologies are direct or indirect.

Finally, an independent study by Bonora et al. presented the

in vivo production of d4T to ZDV in plasma from patients treated with ZDV (3). However, these findings are in complete discrepancy with those of Becher et al., since the latter group could not measure d4T in plasma from those patients showing the intracellular ZDVTP transformation (2). It is worth noting that Bonora et al. conducted therapeutic drug monitoring (TDM) for the simultaneous detection of all licensed antiretroviral agents. ZDV plasma determination from HIV patients was conducted over the same period that d4T plasma TDM determination was performed. In the Bonora et al. study, the d4T levels measured had no correlation with ZDV levels in ZDV-treated patients. More importantly, three subjects under ZDV treatment were found to have up to 30-fold d4T relative to ZDV plasma concentration. Taking into consideration the findings reported by King et al., care should be taken in the laboratory when conducting simultaneous protocols at the same laboratory.

In conclusion, the points raised by Benech et al. in their correspondence give little insight into the contradictory results for the *in vivo* transformation of ZDVTP to d4TTP between the two studies. However, we are of the disposition to exchange samples between laboratories, as suggested by Benech et al., in order to clarify this issue.

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