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Design, synthesis and studies of triphosphate analogues for the production of anti AZT-TP antibodies

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ABSTRACT

Triphosphates anabolites are the active chemical species of nucleosidic reverse transcriptase inhibitors in HIV-therapy. Herein, we describe (i) the design of stable triphosphate analogues of AZT using molecular modelling, (ii) their synthesis and (iii) their use for producing anti AZT-TP antibodies in the aim of developing an immunoassay for therapeutic drug monitoring.

Nucleoside triphosphates are important metabolites in many cellular processes, and analogues of these compounds have major therapeutic applications in HIV. The triphosphate metabolites (NRTI-TP) of the antiviral analogues are the active chemical species. They compete into infected cells with endogenous nucleotides for incorporation into the replicating HIV DNA, which results in termination of viral replication. Great interindividual variability in the production of phosphorylated metabolites, as well as poor correlation between plasma drug levels and therapeutic outcome, emphasize the need for easy routine quantification methods of triphosphate metabolites. The ability to detect and quantitate the NRTI-TP concentrations in infected cells will lead to a better understanding of the pharmacology of these agents and facilitate the discovery of improved therapeutic approaches.

However the monitoring of nucleoside metabolites in treated patients requires highly sensitive analytical methods and so far few methods have been published using mainly direct LC–MS/MS detection techniques[1,2] or indirect dephosphorylation methods.[3,4] Despite the advantages of LC–MS/MS, this method is hindered by high technical requirement and cost of use. In our aim to develop antiviral enzyme immunoassays[5,6] (EIA), we were interested in the development of a general strategy for direct measurement of triphosphorylated analogues. The objective is to establish competitive EIA based on the use of specific rabbit polyclonal antibodies (pAb). Indeed EIA are potentially very sensitive, and these techniques present the valuable advantage to be directly and easily applicable to clinical routine analysis of large series of samples. Herein we described our study for the production of antibodies directed against the triphosphorylated form (AZT-TP) of the most widely used nucleoside inhibitor in AIDS therapy, AZT (Fig. 1). The synthesis of several triphosphorylated analogues of AZT and the characterization of the corresponding antibodies are presented.

Previous attempts to generate specific and sensitive pAb against triphosphate nucleosides using direct linkage to the triphosphate or isostere were not fully successful.[7,8] Therefore, we tried to evaluate a systematical approach which combines both novel strategies and adapted concepts that had been successful for the synthesis of triphosphate analogues in previously reported studies.[9,10]

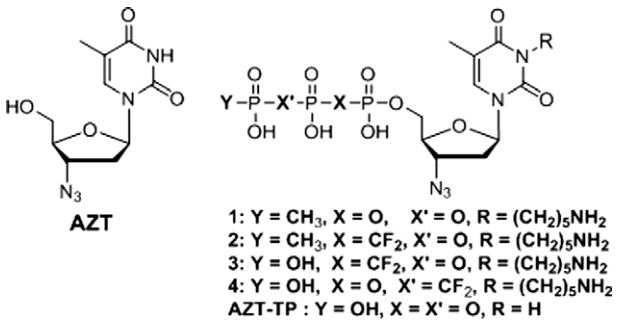

Figure 1. AZT, AZT-TP and AZT-TP analogues.

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One of the crucial steps of the EIA elaboration process is the production of antibodies allowing a very specific detection associated with high sensitivity. As small molecules, AZT-TP analogues are haptens, not immunogenic per se. Thus generating pAb against small haptenic molecules requires their covalent linkage to a larger immunogenic protein carrier by the intermediary of a spacer arm in order to trigger off the immune response. The mode of attachment should be such that the relevant structural determinants of the analyte are free to interact with the immune system. Following successful protocol in the obtention of specific monophosphorylated AZT antibodies[11], we decided to introduce the spacer arm on the nucleobase (Fig. 1). Furthermore in the case of the triphosphorylated form, another major difficulty raises from both the chemical and the enzymatic instability of pyrophosphate bridges leading to a rapid hydrolysis of AZT-TP into AZT in recipient animals. This strong instability could impair the achieving of specific pAb directed against the AZT-TP. To avoid this problem, we decided to synthesize different isosteric and isoelectronic AZT-TP analogues. Several groups have previously reported the synthesis of triphosphate mimics in which one or two bridging oxygen of triphosphate is replaced by methylene, halomethylene, or imido groups.[9,10,12] As expected, a single bridge modification on a triphosphate moiety can enhance the stability of theses analogues to dephosphorylating enzymes.[9] The substitution of the γ-phosphate by a γ-methylphosphonate as well as a bridging oxygen by a CF_2 group on AZT greatly improved the half-life in human blood serum.[10] Based on the literature data, four different mimics in which the γ-phosphate has been replaced by a γ-methylphosphonate and/or the first or second bridging oxygen by a CF_2 group have been designed (Fig. 1).

Moreover, antigen–antibody recognition is based on steric criteria and interactions resulting from the electronic properties of the molecules. The triphosphate mimics should preserve as much as possible the electronic distribution and the spatial conformation of the target compound AZT-TP. Previous reports suggest that the steric and pK_a between difluoromethylphosphonates and phosphates is quite similar. In order to support the above observations in terms of molecular conformation and charge distributions, a molecular modelling study of the compound **1** to **4** without the spacer arm (R = H) was preliminary carried out (see Supplementary data).

A structural analysis of the five structures illustrated in Figure 2 was performed. The average gyration radius data (4.4, 4.8, 6.1, 5.4 and 4.5 Å for AZT-TP, **1**, **2**, **3** and **4**, respectively) suggest that all isosteres are less globular than AZT-TP. Compounds **1** and **4** showed however shapes closer to that of AZT-TP while **2** and **3** exhibited slightly more elongated structures, probably due to the CF_2 group between the α and β phosphates groups. On the contrary, the lack of CF_2 group naturally makes **1** behave like the original AZT-TP, with a closer distance between the γ unit in the phosphate tail and the thymine residue. **4** recovers however a closer interaction between these two moieties suggesting that a CF_2 group between the β and γ units of the triphosphate tail does not strongly affect the triphosphate orientation with respect to the thymine base.

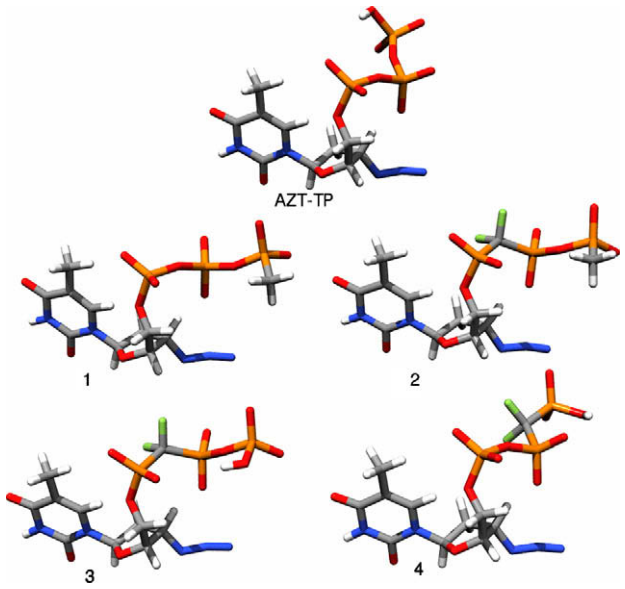

Figure 2. Typical 3D-structures of AZT-TP and 1,2,3,4 compounds. The atom colour is as follows: C, grey; O, red; N, blue; H, white; P, orange and Cl, green.

The results presented above prompted us to synthesize the target haptens **1–4**. The spacer arm on AZT was introduced first to give a common intermediate **5**.[11] Beside the fact that the four triphosphate analogues can be prepared from **5**, introduction in an earlier step of the lipophilic side chain is advantageous since it makes the AZT derivatives more soluble in organic solvents and thus easier to handle and to purify.

The triphosphate analogues **11–13** required for the preparation of haptens **1–3** were synthesized in three steps starting from **5** according to a modified procedure adapted from Blackburn[13] (Scheme 1).

The 5′-hydroxyl group of **5** was first activated by a tosylate group and then reacted with the tristetrabutylammonium salt of pyrophosphate[14] or difluoromethylenediphosphonate[10] to give after ion-exchange chromatography the diphosphate analogues **9** and **10** in 43% and 38% yield, respectively. The reaction was performed with an excess of the pyrophosphate and diphosphonate salts (5–7 equiv) to limit the formation of the dimer and at 60 °C to increase the reaction rate (1 h 30 min at 60 °C, 5 days at room temperature). The triphosphate derivatives **11** and **12** were obtained by a procedure used for the preparation of nucleoside γ-substituted triphosphates. Reaction of **9** and **10** with methylphosphonic dichloride, triazole and triethylamine gave after ion-exchange chromatography compounds **11** and **12** in 49% and 43% yield, respectively. The same procedure was applied for **13** (29% yield) using phosphorus oxychloride instead of methylphosphonic dichloride. The connection between the phosphates of **11** and **12** was further confirmed by 2D ^{31}P–^{1}H NMR which showed correlations between α-P and H-5′, and γ-P and terminal CH_3.

The triphosphate analogue **14** required for the preparation of hapten **4** was obtained in 47 % in a one pot synthesis starting from **5** according to the procedure described by Wang.[10]

The final haptens **1–4** were obtained after ammonolysis of compounds **11–14** at 40 °C for 3 h in 90–97% yield. In order to avoid side reactions, the progress of the reaction was monitored by ^{19}F NMR spectroscopy. A characteristic upfield shift was observed between −76.3 ppm and −76.0 ppm upon the cleavage of the trifluoroacetyl group protecting the terminal NH_2 of the spacer arm. After 3 h, removal of the protecting group was completed. Upon these conditions, the integrity of the triphosphate group was unaffected as checked by ^{31}P and ^{19}F NMR spectroscopy. The structures of haptens **1–4** were ascertained from ^{31}P, ^{19}F, ^{1}H NMR and MS analysis (see Supplementary data).

In order to prepare the immunogens, the haptens **1** to **4** were conjugated to BSA with glutaraldehyde to afford **IMU-1** to **4**, respectively. BSA was preferred to keyhole limphet hemocyamin because of its solubility in various aqueous buffer and the possibility to carry out mass spectra analysis on the synthesised conjugates. The molecular weights of the immunogen **IMU-1** to **4** were determined by Maldi-TOF and found to be 78,171 Da, 79,078 Da, 76,958 Da and 78,828 Da, respectively, which represent an average coupling of 17 molecules of hapten per BSA for **IMU-1**, **2** and **4** and 14 for **IMU-3**.

The tracer **T1** to **T4** were obtained by covalently coupling, respectively, the hapten **1** to **4**, previously activated with *N*-succin-