Fluorescent labelling of azidothymidine: introduction to personalised antiviral therapy

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INTRODUCTION

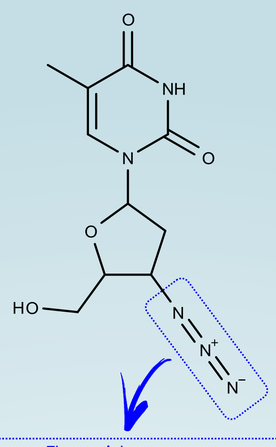
Azidothymidine (AZT, ZDV) is an antiretroviral drug, used as a stand-alone medication or in complex formulations for HIV infection treatment of highly-active anti-retroviral therapy (HAART) compositions.

HAART
HIV/AIDS Alliance

Medication with AZT is associated with many side effects such as myelotoxicity, neutropenia, and hepatotoxicity, which raise concerns about safety of the treatment.
In such situation, adjustment of an individual dose of the drug is highly beneficial for the patient. It is particularly important if the inter-individual differences in the rate of drug metabolism are considered.[1]

Individual profiling of AZT metabolism could be a step towards the treatment of viral infections using the personalized dosage therapy. The therapy has the potential both to reduce mortality and morbidity rates among HIV-infected people, and to improve their quality of life.

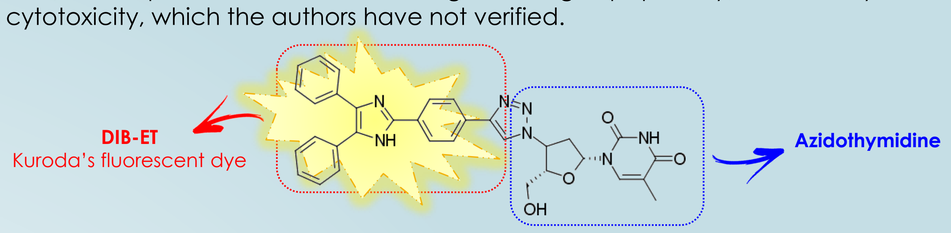
Azidothymidine
HIGHLY ACTIVE ANTIRETROVIRAL THERAPY
nucleoside analog reverse transcriptase inhibitor (NRTI)[2]



The azide group
makes AZT a ready-to-use substrate for the *Copper(I)-Catalyzed Azide-Alkyne Cyloaddition* (CuAAC)

CURRENT SOLUTION

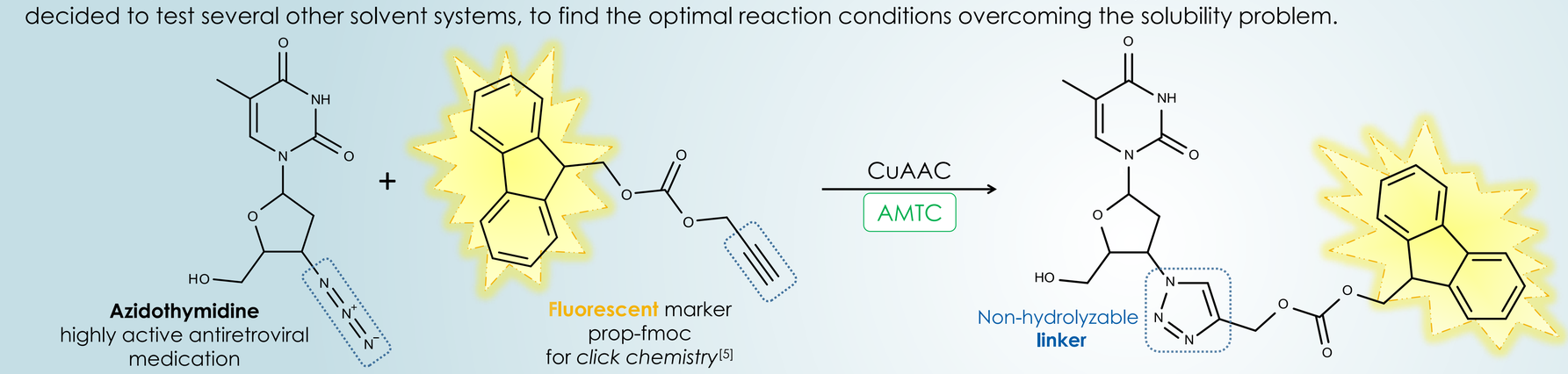
Azidothymidine contains an azide group in its chemical structure. It's fluorescent labeling allows effective analysis of azidothymidine. The proposed fluorescent conjugation methodology is based on the copper(I) catalyzed azide-alkyne cycloaddition (CuAAC), which is the main reaction of the *click chemistry* approach. This strategy focuses on the use of efficient and easy to perform reactions and is widely used in pharmaceutical sciences and fluorescent labelling.[3]
In 2014 Kuroda and co-workers developed novel fluorescent derivatization method using a fluorescent alkyne probe DIB-ET, for chromatographic analysis of azide compounds, including azidothymidine. Their method enabled sensitive and selective determination of azidothymidine in rat plasma samples without interference from biological components. However, DIB-ET probe has 4 aromatic rings and high lipophilicity, which may indicate high cytotoxicity, which the authors have not verified.



For the AZT-DIB-ET conjugate, a RP-HPLC (reversed phase-high-performance liquid chromatography) analysis method were performed, using fluorometric detection. The LOD and LOQ values estimated by Kuroda *et al* for the conjugate were respectively $1.0 \cdot 10^{-9}$ mol/l and $3.3 \cdot 10^{-9}$ mol/l.[4]

FLUORESCENT LABELLING OF AZIDOTHYMIDINE BY PROP-FMOC

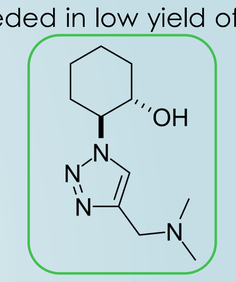
In 2015 we designed and obtained a novel alkyne-containing fluorescent probe, 9*H*-fluoren-9-ylmethyl prop-2-yn-1-yl carbonate (prop-fmoc) for efficient labeling of AZT. Prop-fmoc was prepared easily, in 91% yield, from highly economical and widely applicable common reagents as fmoc-chloride and propargyl alcohol.
The initial cycloaddition reaction between AZT and prop-fmoc performed in the 2:1 water:ethanol system with 10 mol% of copper and 20 mol% of AMTC proceeded in low yield of 21%. Therefore, we decided to test several other solvent systems, to find the optimal reaction conditions overcoming the solubility problem.



Azidothymidine
highly active antiretroviral medication

Fluorescent marker prop-fmoc for *click chemistry*[5]

Non-hydrolyzable **linker**



AMTC is a copper(I)-stabilizing ligand effective for improving the reaction outcome.[6]

International patent application PCT
Derivatives of 1,2,3-triazolyl cyclohhexan-1-ol and its use
P. W. Szafrański, P. Kasza, M. T. Cegła
PCT/PL2016/050026, WO2016/200283 A1, **2016**

Reactions were performed using the $CuSO_4$–sodium ascorbate catalytic system; ligand-free or using 20 mol% of AMTC, and various solvents. The isolated products were analyzed by HPLC-MS to determine their purity. The HPLC yield has been calculated by multiplying the isolated yield by HPLC purity of each sample.

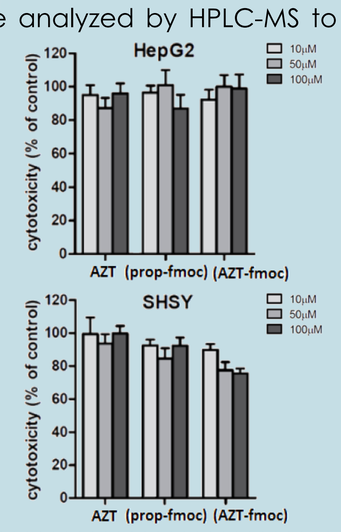
	lignad-free			AMTC		
solvent	**isolated**	**HPLC purity**	**HPLC yield**	**isolated**	**HPLC purity**	**HPLC yield**
water	77%	1,58%	**1%**	76%	6,48%	**5%**
2:1 water:ethanol	93%	26,56%	**24%**	21%*	99%*	**21%**
dichloromethane	65%	98,49%	**64%**	96%	98,41%	**95%**
2:1 water:*t*-butanol	>99%**	15,90%	**16%**	>99%**	92,27%	**91%**
2:1 water:2-propanol	>99%**	33,74%	**34%**	>99%**	82,68%	**82%**

Best synthetic results are for
✓ **dichloromethane**
✓ **2:1 water:*t*-butanol**

**the samples contained residual solvent, difficult to remove

CYTOTOXICITY NOTE

Cytotoxicity studies were carried out during colorimetric assay for assessing cell metabolic activity (MTT assay).
We used neuronal cell lines such as SHSY (human neuroblastoma cell lines) to verify neurotoxicity and human hepatoma cell lines such as HepG2 for hepatotoxicity



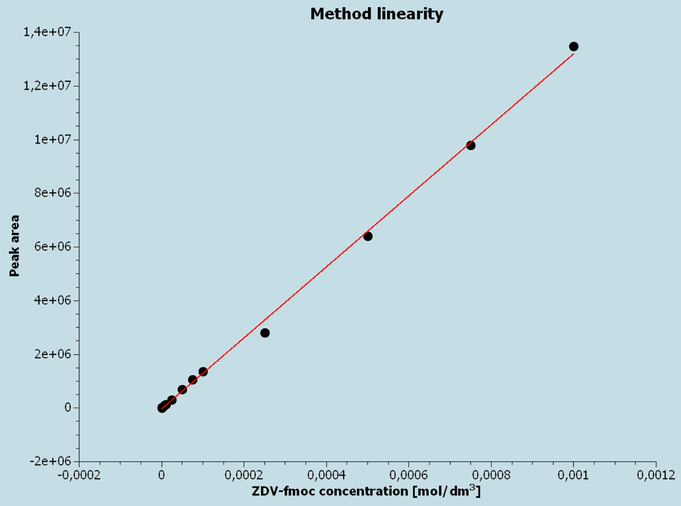
Efficient labelling of azidothymidine with a fluorescent marker would allow to easily determine its concentration in samples, first in model solutions and next, in patient blood and urine samples.[3] This would extend the application of personalized medicine to antiviral treatment with azidothymidine.

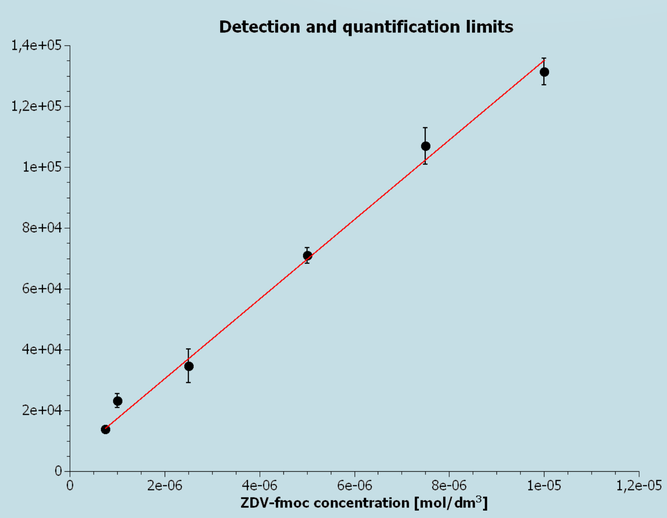
ANALYTICAL RESEARCH RESULTS

Fluorescence-based detection enables efficient analysis of studied compounds, significantly increasing the sensitivity of determinations compared to absorption-based methods.
We compare available in most laboratories adsorption method (HPLC with UV detector) and sensitive fluorescence spectroscopic method (HPLC with fluorescence detector) for determining LOD and LOQ analytical values.

HPLC-UV

Metod linearity is retained at concentrations ranging from **$7.5 \cdot 10^{-7}$ to 10^{-3} mol/dm³** (R^2= 0.99845).

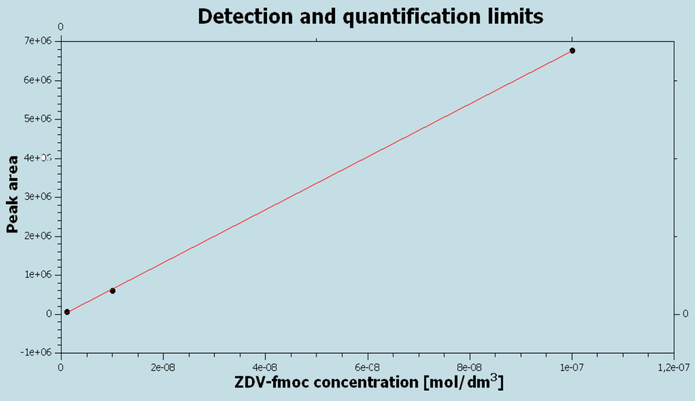




LOD= $8.9 \cdot 10^{-7}$ and **LOQ= $2.7 \cdot 10^{-6}$** (R^2= 0.99928).

HPLC-FLUORESCENCE (preliminary results)

For the AZT-fmoc conjugate, a HPLC analysis method was developed and initial analyses were performed for three concentrations: 10^{-7}, 10^{-8}, 10^{-9} mol/dm³, using fluorometric detection.
The estimated LOD and LOQ for the conjugate were respectively **$2.0 \cdot 10^{-9}$** and **$6.1 \cdot 10^{-9}$ mol/dm³**, which is similar to the results presented by Kuroda *et al* (LOD=$1 \cdot 10^{-9}$ mol/dm³).



The obtained results are a starting point for further research into azidothymidine labeling and the development of universal and effective bioconjugation methodologies.

CONCLUSIONS

Personalization of AZT therapy would involve individual adjustment of the azidothymidine dose used in treatment by monitoring azidothymidine concentrations in patients' blood.
Such an approach could result in clear and precise determination of the patient's metabolic profile, minimizing side effects and maximizing the therapeutic effect during pharmacotherapy.

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[6] P.W. Szafrański, P. Kasza, M.T. Cegła, *Tetrahedron Lett.* **2015**, 56, 6244-6247.