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Synthesis and sensing properties of a new carbazole fluorosensor for detection of abacavir

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An abacavir-targeted fluorosensor based on the carbazole moiety has been synthesised and characterised. Recognition of abacavir is by base pairing between a uracil moiety present in the fluorosensor and the guanine moiety of abacavir. The fluorosensor exhibits five-fold quenching in the presence of 50 μM abacavir. Its sensitivity to abacavir is superior to that of other reverse transcriptase inhibitors: zidovudine, lamivudine and didanosine. Due to its high sensitivity, this fluorosensor has the potential to be used in multi-analyte array-based detection platforms as well as in microfluidics systems.

Keywords: antiretroviral drugs; base pairing; fluorescence spectroscopy

Various antiretroviral drugs are currently prescribed for the treatment of patients infected with human immunodeficiency virus (HIV) (*1–3*). Of those patients undergoing antiretroviral chemotherapy, over 90% of them are prescribed the antiretroviral drug abacavir. However, one of the major drawbacks associated with the administration of abacavir is a 90% increase of the risk of heart attack (*4–6*). Therefore, low dosages per outcome ratios are of paramount importance. The ability to monitor antiretroviral drug concentrations on an individual basis will allow practitioners to create a patient-specific regimen to reduce individual suffering. This would also be particularly relevant to child patients, where the clearance rate for drugs like abacavir has been reported to be twice that of adults (*7*, *8*).

Typically, concentrations of antiretroviral drugs, such as abacavir, are determined by the use of liquid chromatography, mass spectrometry or a combination thereof (*9–12*). However, these methods are typically slow, expensive and require a high degree of experimental expertise. A combination of these methods with optical techniques such as absorbance or fluorescence detection has also been reported (*13–15*), and in a few cases, electrochemical detection methods have also been applied (*16*, *17*). However, the increased use of fluorescence spectroscopy for detection of antiretroviral drugs would introduce numerous advantages, as fluorescence-based techniques have higher sensitivity when compared to absorbance techniques, and are more specific and less susceptible to interferences due to low concentrations of the fluorosensor and/or analyte. When compared to other analytical techniques, such as gas chromatography or HPLC, fluorescence-based techniques can also exhibit increased cost efficiency.

Here, we present a carbazole fluorosensor possessing two uracil moieties that have an affinity towards the nucleoside analogue reverse transcriptase inhibitor class of antiretroviral drugs by mimicking purine base pairing. We chose carbazole as the signal transduction unit because of its chemical stability, large Stokes shift and high fluorescence quantum yield. Furthermore, carbozole can be readily functionalised in such a way that enables its immobilisation in polymer layers and nanoparticles (*18*, *19*).

The two major steps involved in the synthesis of the carbazole-based chemosensor are presented in Scheme 1. Firstly, 3,6-dibromo-9-octyl-9H-carbazole (**1**) was dissolved in tetrahydrofuran and cooled to − 78°C. Then, *sec*-butyllithium and 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (ITD) were added to obtain 3,6-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*N*-octylcarbazole (**2**). The bis-uracil-carbazole **3** was obtained by mixing **2** with two equivalent amounts of 5-bromouracil (5-BU) in toluene, in the presence of tetrakis(triphenylphosphine)palladium and sodium carbonate. The reaction was carried out under reflux in an argon atmosphere.

The fluorescence spectra of the fluorosensor, which was gradually quenched with increasing concentrations of abacavir, are shown in Figure 1. A decrease in fluorescence intensity is observed upon the addition of up to 50 μM abacavir. The quenching is caused by electron transfer from the antiretroviral drug to the carbazole

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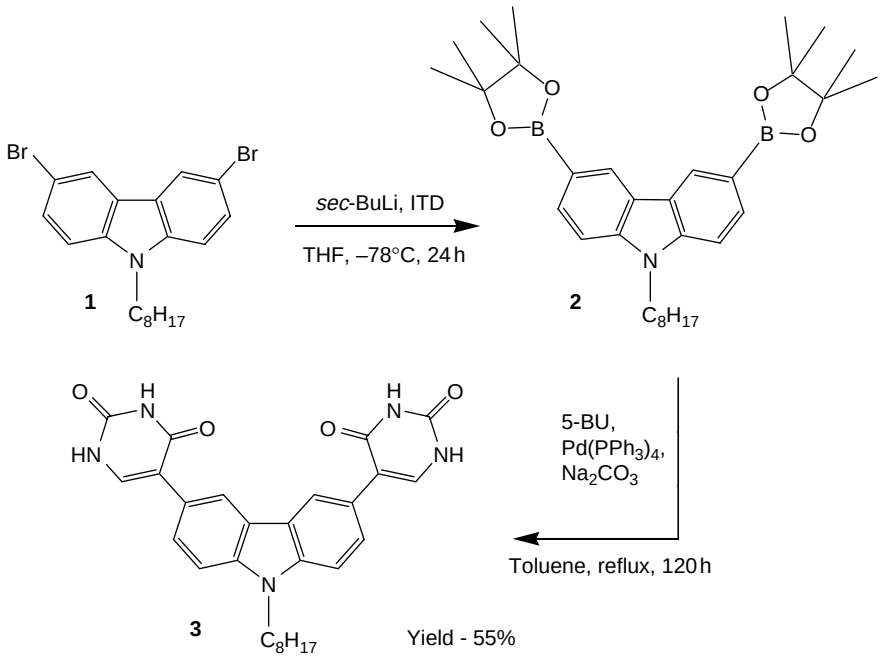

Scheme 1. Design of the bi-uracil-carbazole fluorosensor.

moiety upon triple hydrogen bonding with the uracil groups. The fluorescence lifetime of the fluorosensor was unchanged over the complete abacavir concentration range. This gives very good evidence that the fluorescence quenching is of static character, caused by the formation of a stable fluorosensor–drug complex in the ground state (*20*).

In order to evaluate the selectivity of the fluorosensor, it was tested towards other reverse transcriptase inhibitors: didanosine, zidovudine and lamivudine. The rationale behind this choice is based on the fact that zidovudine and lamivudine are commonly administered in combination with abacavir in highly active antiretroviral therapy (HAART). Therefore, they can be considered as competitors to abacavir binding.

The fluorescence quenching of the sensor can be attributed to the formation of a stable ground state complex between **3** and the abacavir molecule, due to hydrogen bonding. Consequently, this fluorescence quenching can be described by the Stern–Volmer (S–V) theory (*20*). S–V plots corresponding to each drug are presented in Figure 2. The plots refer to the relationship between the fluorescence quenching of the fluorosensor and different concentrations of abacavir, zidovudine, lamivudine and didanosine, in buffered aqueous solutions.

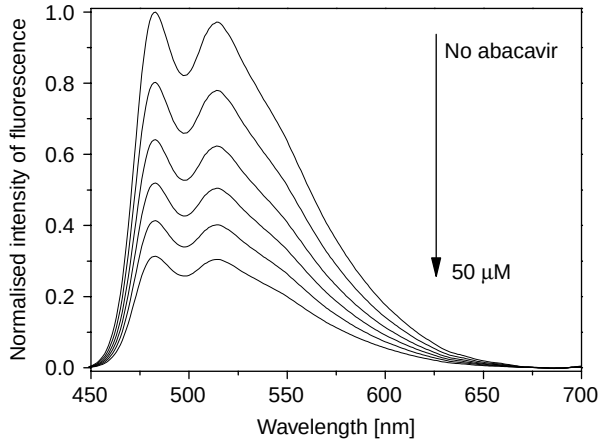

Figure 1. Fluorescence quenching of the fluorosensor with increasing concentrations of abacavir in 0.1 M aqueous HEPES buffer at pH 7.4 ($\lambda_{exc} = 360$ nm).

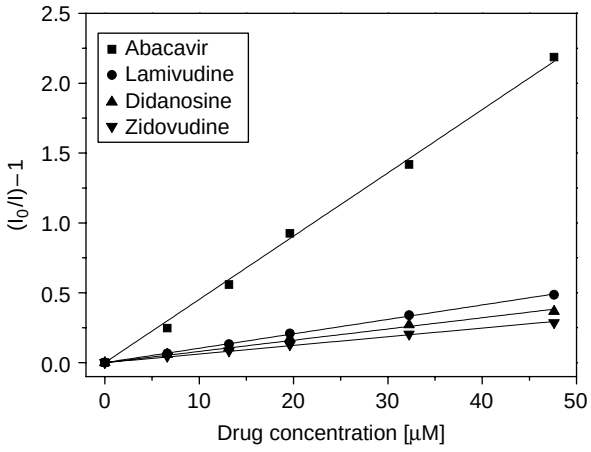

Figure 2. S–V plots for the studied drugs determined by steady-state fluorescence spectroscopy.

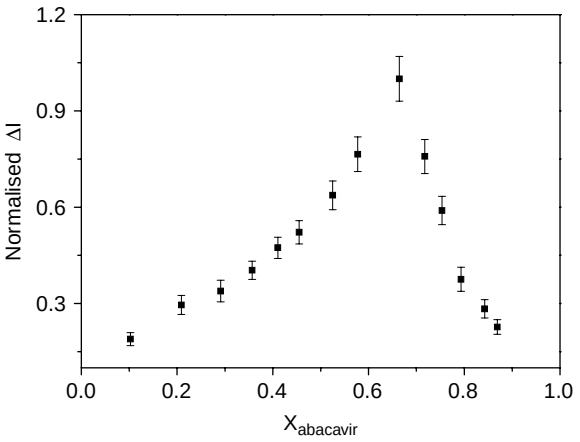

Figure 3. Job plot for the complexation of the carbazole fluorosensor with abacavir in 0.1 M aqueous HEPES buffer at pH 7.4 ($\lambda_{exc} = 360$ nm). The data presented are an average of 300 measurements.

The S–V constants were found to be $4.6 \times 10^4\,M^{-1}$ for abacavir, $1 \times 10^4\,M^{-1}$ for lamivudine, $0.8 \times 10^4\,M^{-1}$ and $0.6 \times 10^4\,M^{-1}$ for zidovudine, respectively. This means the fluorosensor is five times more sensitive to abacavir than to the other nucleoside reverse transcriptase inhibitors usually administered within the HAART regimen.

We attribute this enhanced selectivity of the fluorosensor to abacavir to the presence of guanine within its structure. It has already been shown by NMR spectroscopy that guanine binds to uracil, via triple hydrogen bonding, more strongly than with thymine, cytosine or adenine (*21*, *22*). In the case of thymine and cytosine, only weak hydrogen bonds are observed; and with adenine, uracil is only capable of forming a wobble base pair. The fluorosensor was also tested against metal ions such as Na^+, K^+, Ca^{2+} and pyrophosphate (PP). Quenching caused by PP (150 mM) was 5% of that caused by abacavir. For Na^+ and K^+, it was 10%, while for Ca^{2+} it was 18%, probably due to stronger ion coordination. When the fluorosensor had already formed hydrogen bonds, for example in the presence of abacavir, no further effect was observed. The fluorosensor had preferentially bound with the drug over the metal ion or phosphate. The influence of pH on the fluorescence of the fluorosensor in the presence, and in the absence, of abacavir was also determined. For this purpose, the fluorescence of the fluorosensor was collected before and after the addition of 50 μM abacavir in 0.1 M aqueous HEPES buffered solutions, in the pH range from 5.0 to 8.0. A slight loss of sensitivity was observed at lower pH values, with a 10% reduction at pH 5.0 compared to that at pH 7.4. This feature might be attributed to the partial protonation of the tertiary amino group present in the carbazole moiety. Its uncomplexed pK_a value was around 5.8 (*23*). Nevertheless, changes in intensity were still readily noticeable in the presence of abacavir. Moreover, to confirm the formation of the complex between the fluorosensor and abacavir, we performed titration experiments using steady-state fluorescence spectroscopy. The Job plot for the titration of **3** with abacavir is shown in Figure 3. One can easily distinguish the formation of a complex with a 1:2 stoichiometry, which supports our previous observations concerning the mechanisms of complex formation.

In humans, after the administration of a 600 mg oral dose of abacavir, the concentration of abacavir and its metabolites typically ranges from 0.500 to 8000 ng/mL (*24*, *25*). In some cases, values in the range of 50–1200 ng/mL in human plasma were reported (*26*). In order to verify whether our sensor is able to detect the antiretrovirals used in this range, we determined the limits of detection (LODs) for each drug. The LODs were calculated by dividing three times the standard deviation of the fluorescence signal at zero concentration with the slope of the quenched fluorescence as the concentration of the drug increases.

The LOD for abacavir was found to be 200 ng/mL (4.2 μM), which is advantageously below the concentration of abacavir usually found in human plasma. The LODs for lamivudine, didanosine and zidovudine were found to be 1.1 μg/mL (24 μM), 1.45 μg/mL (30 μM) and 1.85 μg/mL (40 μM), respectively. We also tested the sensor's ability to detect abacavir in a mixture of these antiretroviral drugs. To a solution containing 25 μM lamivudine, 25 μM atazanavir and 10 μM ritonavir, up to 50 μM abacavir was added. Under these conditions, the LOD for abacavir was 500 ng/mL, which supports the practical usefulness of the fluorosensor.

In conclusion, a carbazole fluorosensor for antiretroviral drugs based on a uracil moiety was successfully synthesised. The sensor exhibits high sensitivity towards abacavir, and moderate sensitivity to didanosine, zidovudine and lamivudine. Due to its low LODs, the fluorosensor has the potential to be applied in multi-analyte array-based detection platforms, as well as in microfluidics systems. Because of the well-known electrochemical properties of carbazole, the fluorosensor we developed could also be used for the determination of antiretroviral drugs by electrochemical methods. Furthermore, the versatile chemistry of carbazole allows for the introduction of other functional groups (such as amino, carboxylic or mercapto functional groups) to the sensor molecule, which might be subsequently used for the covalent immobilisation of the fluorosensor onto surfaces of nanoobjects or biomolecules.

Experimental section

Abacavir was purchased from Sequoia Research Products (Pangbourne, UK), while didanosine was obtained from TCI Europe (Eschborn, Germany). All other chemicals

were purchased from Sigma-Aldrich (München, Germany) and used without any further purification. All solvents, both for chromatography and spectroscopy, as well as labware, were from Roth (Karlsruhe, Germany). ^{1}H NMR spectra were recorded in deuterated chloroform ($CDCl_3$) on a Brucker 250 spectrometer. Chemical shifts (δ) are denoted in ppm and were referenced to internal tetramethylsilane (0.0 ppm). The splitting patterns are designated as follows: (s) singlet, (d) doublet, (t) triplet, (quin) quintet and (m) multiplet. Preparative column chromatography was carried out on glass columns of different sizes packed with silica gel Merck 60 (0.035–0.070 mm; Darmstadt, Germany). Mass spectra were recorded on a MAT SSQ 710 from Thermo Electron Finnigan (Bremen, Germany). The electronic absorbance and fluorescence spectra were recorded on a Lambda UV/vis spectrophotometer (Perkin Elmer, Rodgau, Germany) and a Fluoromax 3 spectrophotometer (Horiba Jobin Yvon, Unterhaching, Germany), respectively. For the purposes of time-resolved fluorescence spectroscopy, the frequency-doubled output of a Titan Sapphire laser (Tsunami 3960; Spectra Physics, Santa Clara, CA, USA) was used to excite samples. The luminescence was detected in a right angle configuration to the incoming beam. A multichannel plate (ELD EM1-132/300; Europhoton GmbH, Berlin, Germany) coupled to a FL920 fluorescence lifetime spectrometer (Edinburgh Instruments, Livingstone, UK) was used for the signal detection. The time-resolved emission was collected in the time-correlated single photon counting mode. All optical measurements were done at a temperature of 25 ± 1°C. A 50 μL aliquot of a previously prepared solution of compound **3** (5×10^{-4} M in dimethylformamide) was dissolved in 5 mL of 0.1 M aqueous HEPES buffer at pH 7.4 (5×10^{-6} M, optical density ≤ 0.1), and then 3 mL of this solution was transferred to a 1 cm × 1 cm quartz cuvette (Hellma GmbH, Jena, Germany). Small aliquots (10–300 μl) of the analyte were then added to the cuvette. The pH value remained constant throughout all the measurements, as measured using a pH meter (Hanna Instruments, Woonsocket, MI, USA).

Synthesis of 3,6-bis(5-uracilyl)-N-octylcarbazole (3)

For the preparation of 3,6-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*N*-octylcarbazole (**2**), *N*-octylocarbazole (**1**) (0.874 g, 2.0 mmol) was dissolved in 100 mL of absolute tetrahydrofuran under argon atmosphere. This solution was cooled to − 78°C before 3.43 mL of *sec*-BuLi (1.4 M in hexane, 4.8 mmol) was added. The reaction mixture was stirred for 20 min and ITD (0.98 mL, 4.8 mmol) was then added. After 24 h, the reaction mixture was allowed to warm up to room temperature and 50 mL of water was then added. The solution was extracted with 3 × 50 mL portions of ethyl acetate. The combined organic layers were washed with 20 mL of brine, dried over $MgSO_4$ and evaporated to brown oil. The product was purified by column chromatography (eluent: hexane–AcOEt, 9:1). The yield, after purification by column chromatography, was 0.87 g (82%) of a light yellow solid. *3,6-Bis(5-uracilyl)-N-octylcarbazole* was then prepared by mixing **2** (0.4 g, 0.75 mmol) with 5-BU (0.403 g, 2.1 mmol), $Pd(PPh_3)_4$ (138 mg, 0.12 mmol) and 2 M Na_2CO_3 aqueous solution (20 mL, 40 mmol) in toluene (100 mL), and stirring at reflux under an argon atmosphere for 120 h. Water (150 mL) and $CHCl_3$ (150 mL) were then added. The organic phase was separated and the water phase was extracted with 3 × 50 mL of chloroform. Organic phases were collected and washed with water, then with brine solution, dried over anhydrous $MgSO_4$, filtered, and the solvents left to evaporate off. The crude product was purified over a chromatographic silica gel column (hexane–AcOEt, 3:1) to give **3**. 3,6-Bis(5-uracilyl)-*N*-octylcarbazole, black heavy oil (207 mg, 55%). (250 MHz, $CDCl_3$): ^{1}H NMR $\delta = 7.73$ (d, $J = 8.0$ Hz, 2H); 7.55–7.42 (m, 2H); 7.39–7.28 (m, 4H); 4.33 (t, $J = 7.2$ Hz, 2H); 1.98–1.83 (m, 2H); 1.50–1.19 (m, 10H); 0.88 (t, $J = 6.6$ Hz, 3H). Elemental analysis for $C_{28}H_{29}N_5O_4$ was calculated to be: C, 67.32; H, 5.85; N, 14.02; O, 12.81; in actuality, it was measured to be: C, 69.47; H, 5.55; N, 14.18; O, 12.53; molecular mass, as measured by mass spectroscopy using micro-electrospray ionisation, was 507.2 (M − H). Quantum yield was 0.4. The extinction coefficient (ε) measured at $\lambda = 360$ nm for the finished fluorosensor yielded 36,000, while for abacavir it was equal to 100.

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