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# Benzothiophen-pyrazine scaffold as a potential membrane targeting drug carrier

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ABSTRACT

The fluorescent properties of 2,5-di(benzo[b]thiophen-2-yl)pyrazine as a potential membrane targeting drug carrier were characterized and it was shown that its fluorescence intensity was much higher in organic solvent than in water. The embedding of studied compound by liposomes leads to ca. 2 orders of magnitude increase in its fluorescence intensity, suggesting its preferential accumulation in membranes. Preliminary biological studies showed its ability to accumulate in cells, and the concentration of 10 μM was sufficient for homogeneous staining of cells. The treatment of mouse carcinoma CT26 cells with studied compound up to 200 μM resulted in decreasing of viable cells by ca. 30%. Its reactivity towards albumin was found to be moderate with an association constant of $6\times10^{4}\,M^{-1}$, while no interaction with DNA was observed. Our findings encourage for further studies on functionalization of this molecule to obtain a new class of anticancer drugs targeting membrane.
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## 1. Introduction

Recently a lot of attention has been devoted for identification of specific targets (receptors) expressed on cancer cells, but rather little work has been dedicated for targeting the cancer cell membranes *per se* [1]. The former concept is based on deactivation of a specific protein or process/signaling pathway. The latter approach comprises designing of drugs, which can cause the permeabilization/disruption of plasma or mitochondrial membranes in cancer cells. The selectivity can be reached e.g. by taking advantage of acidic environment of solid tumor, attaching additional molecules (i.e. folic acid) which have receptors on tumor cells or others.

Previously, we have described the synthetic route to 2,5-di(benzo[b]thiophen-2-yl)pyrazine (*bis*-BTpyr, Fig. 1) which is characterized by high fluorescence quantum yield in chloroform [2]. Due to its very high lipophilicity ($\log P=5.25\pm0.56$) [3,4] it might be expected that this type of compounds will be preferentially accumulated in the cell membrane. Both findings encouraged us for further studies on its physicochemical and photophysical properties in the context of its application in biological systems. Given that 2,5-di(benzo[b]thiophen-2-yl)pyrazine derivatives are slightly soluble in water, a carrier in human body is in fact necessary. Human serum albumin (HSA) is postulated to be a natural carrier for drugs and it has been shown that high amounts of albumin tends to accumulate in malignant cell where it gets metabolized [5,6]. In this context we have investigated the interaction of *bis*-benzothiophen-pyrazine scaffold with bovine serum albumin (BSA) which possesses a structural homology with HSA and is often used as a standard for such studies. The model liposomes made from 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and cholesterol (10:1) were used for investigating the ability of the studied compound to accumulate in lipids. The potential interaction of the studied compound with DNA was also explored with the aim to exclude its mutagenic or genotoxic properties. Furthermore, the preliminary biological tests including cytotoxicity were conducted for evaluating the potential application of the studied compound.

## 2. Materials and methods

### 2.1. Sample preparation for physicochemical measurements

2,5-di(benzo[b]thiophen-2-yl)pyrazine (*bis*-BTpyr) was synthesized according to published procedure [2] and its purity was checked by $^{1}$H NMR and HRMS, giving satisfactory results. The stock solution of b*is*-BTpyr ($2.9\times10^{-3}$ M) was prepared in DMSO and for all experiments small volumes of this solution were added into aqueous medium (content of DMSO was kept less than 2%). The stock solution of calf thymus DNA (ct-DNA, Sigma-Aldrich)

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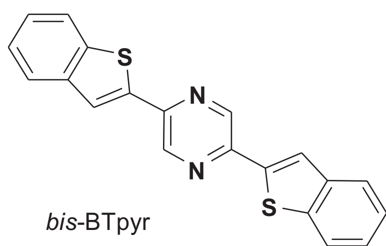


**Fig. 1.** Structure of 2,5-di(benzo[b]thiophen-2-yl)pyrazine.

was prepared by dissolving solid ct-DNA in water and concentrations per nucleotide were determined spectrophotometrically using $\varepsilon_{260}$ of 6600 $M^{-1}$ $cm^{-1}$. The concentration of 1% aqueous solution of ethidium bromide (EB, Sigma-Aldrich) was verified spectrophotometrically ($\varepsilon_{480}$=5450 $M^{-1}$ $cm^{-1}$). The concentration of bovine serum albumin (BSA, Sigma-Aldrich) was determined spectrophotometrically ($\varepsilon_{280}$=44,000 $M^{-1}$ $cm^{-1}$). Reactivity towards biomacromolecules was measured in phosphate buffer solution, PBS (0.05 M, pH 7.4). POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine) and cholesterol were purchased from Sigma-Aldrich and used for preparation of liposomes by the ethanol injection method. The stock solution of lipids was prepared in ethanol by dissolving POPC and cholesterol at a molar ratio of 10:1. Ethanolic solution of lipids was injected rapidly into the previously equilibrated water at 50 °C. The percent of ethanol in solution was less than 2% (v/v). In the experiments fixed concentration of lipids ([POPC]=$30 \times 10^{-6}$ M) and various amount of *bis*-BTpyr were used. All experiments were performed with freshly prepared liposome suspensions and *bis*-BTpyr solution.

### 2.2. Procedures and apparatus for physicochemical measurements

The fluorescence spectra were recorded on Perkin Elmer LS55 spectrofluorimeter. The fluorescence quantum yield was measured using quinine sulfate as a reference. Mean value of minimum three independent experiments was calculated and SD was denoted. The fluorescence lifetime was measured using a time-correlated-single-photon counting (TCSPC) method on Fluorolog-3, Horiba Jobin Yvon spectrophotometer. UV–vis spectra were recorded using a Perkin Elmer Lambda35 spectrophotometer equipped with an Peltier thermostat ($\pm$0.1 °C), while for the measurement of melting temperature of ct-DNA ($T_m$) the in-cuvette temperature sensor was used. The melting temperatures of ct-DNA alone ($3.0 \times 10^{-5}$ M) and in the presence of *bis*-BTpyr ($1.0 \times 10^{-5}$ M) were determined by monitoring the changes in the absorption at 260 nm as the function of temperature ranging from 30 to 95 °C. The experiments were performed in PBS buffer pH 7.4 in the presence of 0.1 M NaCl. The fluorescence spectra in the range of 400–700 nm for different concentrations of *bis*-BTpyr in water, DMSO and liposome suspension were recorded on Tecan Infinite200 Reader using 96 wells plate upon excitation at 370 nm. All measurements were conducted at 37 °C and a quick shaking (ca. 3 s) before measurement was implemented.

### 2.3. Biological studies

Murine colon carcinoma cell line (CT26) was cultured in DMEM medium supplemented with bovine/ferine serum (10%) and antibiotics (1%) at atmosphere of 5% $CO_2$ at 37 °C. Cells for fluorescence imaging were seeded on 96 wells plate at a density of ~30,000 cells/well and cultured for 1 day. *bis*-BTpyr was dissolved in DMSO and diluted in PBS with $Ca^{2+}$/$Mg^{2+}$ to the required concentration (0–$200 \times 10^{-6}$ M) immediately prior to addition into the wells. The final DMSO concentration was kept at 0.5% v/v level, since DMSO $IC_{50}$ for CT26 cell line is 5% (results not shown). After 24 h incubation the tested compound was precisely washed out and cells were kept at PBS solution for imaging. An Olympus fluorescence microscope IX51 equipped with XC10 camera was used for the imaging of *bis*-BTpyr embedded in CT26 cells using 360–370 nm excitation filter. Cells for fluorescence measurements and cytotoxicity studies were cultured for 2 days. The studied compound at the concentrations between 0 and $200 \times 10^{-6}$ M was incubated for 24 h with cells. After that time the cells were washed with PBS. Then the emission spectra at 400–700 nm obtained upon excitation at 370 nm were recorded on Tecan Infinite200 Reader. Next, PBS was washed out and subsequently 200 μl of fresh medium was applied to allow cells repair existed damages. After 24 h, the medium was replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) dissolved in DMEM (0.5 mg/ml) to determine the cell viability. Two series of minimum two plates were performed and SD of a mean value was calculated.

## 3. Results and discussion

### 3.1. Emission spectra of bis-BTpyr

It has been shown that *bis*-BTpyr dissolved in $CHCl_3$ exhibits efficient fluorescent properties [2]. They have also been checked in other solvents and are summarized in Table 1. Both the emission maximum and fluorescence quantum yield strongly depend on the type of solvent. In organic solvents (non-polar aprotic $CHCl_3$, polar aprotic DMSO and polar protic $CH_3OH$) it exhibits relatively high quantum yield of emission while in aqueous solutions the fluorescence is substantially quenched (Table 1). It must be noted that *bis*-BTpyr has high solubility in chloroform and DMSO, moderate solubility in methanol, while it is very poorly soluble in water. The addition of water to methanolic solution of *bis*-BTpyr even up to 30% does not change its fluorescence efficiency, only maximum of fluorescence is shifted from 442 to 445 nm (results not shown). It points out that quenching of fluorescence in water cannot arise from changes in solvent polarity or its specific effects like formation of hydrogen bonds. Therefore, the observed self-quenching of this fluorophore in water can be a consequence either of aggregation [7] or its decomposition. In order to check the possibility of the decomposition of *bis*-BTpyr after dissolution in water, the concentrated DMSO solution of *bis*-BTpyr ($1.5 \times 10^{-3}$ M) was diluted with water (ratio of DMSO/water was kept the same as for fluorescence measurement i.e. 1/63) and subsequently water was removed by vacuum evaporation. The resulted DMSO solution of *bis*-BTpyr was used for measurement of fluorescence quantum yield which was found to be in the same range (0.19) as for freshly prepared compound in DMSO. Therefore the decomposition of *bis*-BTpyr in aqueous solution can be excluded as a reason for observed very low fluorescence efficiency. The aggregation of *bis*-BTpyr in aqueous solution seems to be the most plausible explanation for its very low quantum yield.

**Table 1**
Maximum emission wavelength ($\lambda_{em}$) and fluorescence quantum yield ($\Phi_f$) measured in different solvents for *bis*-BTpyr upon excitation at 370 nm.

| Solvent | Chloroform | DMSO | Methanol | Water |
|---|---|---|---|---|
| $\lambda_{em}$ (nm) | 421, 439 | 423, 440 | 442 | 560 |
| $\Phi$ | 0.30 ± 0.01 | 0.207 ± 0.002 | 0.074 ± 0.001 | 0.006 ± 0.001 |

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