Supplementary information

for

Comment on "Fluorimetric sensing of ATP in water by an imidazolium hydrazone based sensor" by S. Farshbaf and P. Anzenbacher Jr., Chem. Commun., 2019, 55, 1770

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UV-VIS Spectrophotometry

(A) **(B)**

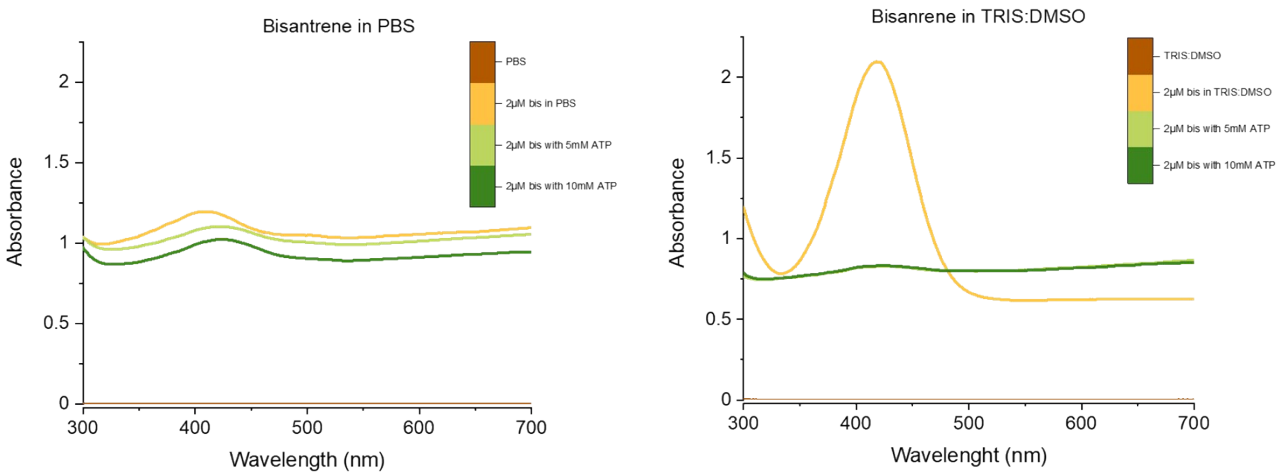

Figure 3: UV-Vis absorbance spectra of bisantrene in PBS and TRIS:DMSO buffer : (a) and (b) Absorbance of different concentrations of bisantrene salt (with and without ATP) at 25˚C in PBS and TRIS:DMSO (90:10) respectively.

Confocal Imaging

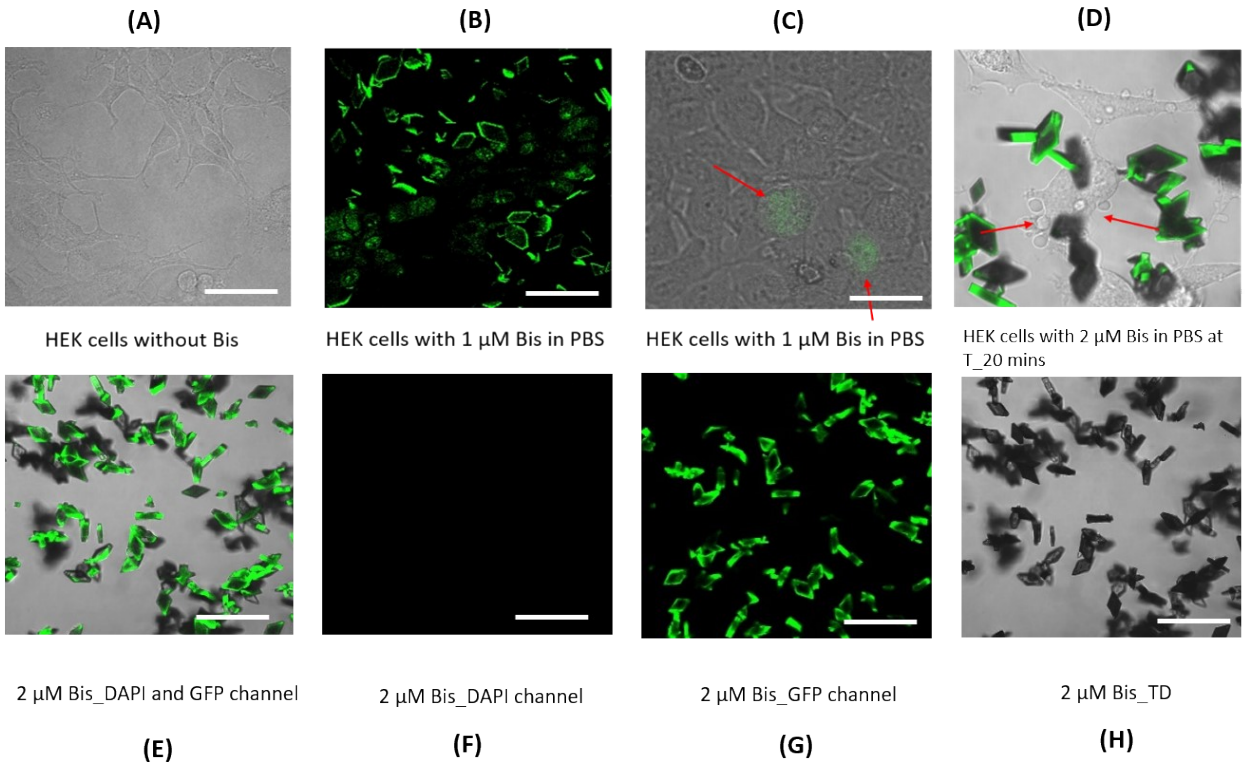

Figure 4: Confocal images of bisantrene salt interaction in HEK cells. (A) Cross-section of HEK cells seeded in 35mm glass-bottom slide and viewed at 60X before addition of bisantrene (t_0 incubation with bisantrene). (B) Fluorescence of 1 µM bisantrene salt in PBS on HEK cells imaged upon excitation at 488nm (t_0 incubation with bisantrene). (C) Uptake of 1 µM bisantrene salt in PBS by HEK cells (red arrows, t_0 incubation with bisantrene). (D) Fluorescence of bisantrene salt imaged upon excitation at 488nm on HEK cells with apoptotic bodies (red arrows) formed after 20 mins of drug addition. (E-G) 2 µM bisantrene crystal solution in PBS. (E) Fluorescence at λ_{ex}=488 nm and λ_{ex}=405nm, (F) Fluorescence at λ_{ex}=405 nm, (G) Fluorescence at λ_{ex}=488 nm, (H) Brightfield. Scale bar: 100 µm.

Polarisation Results

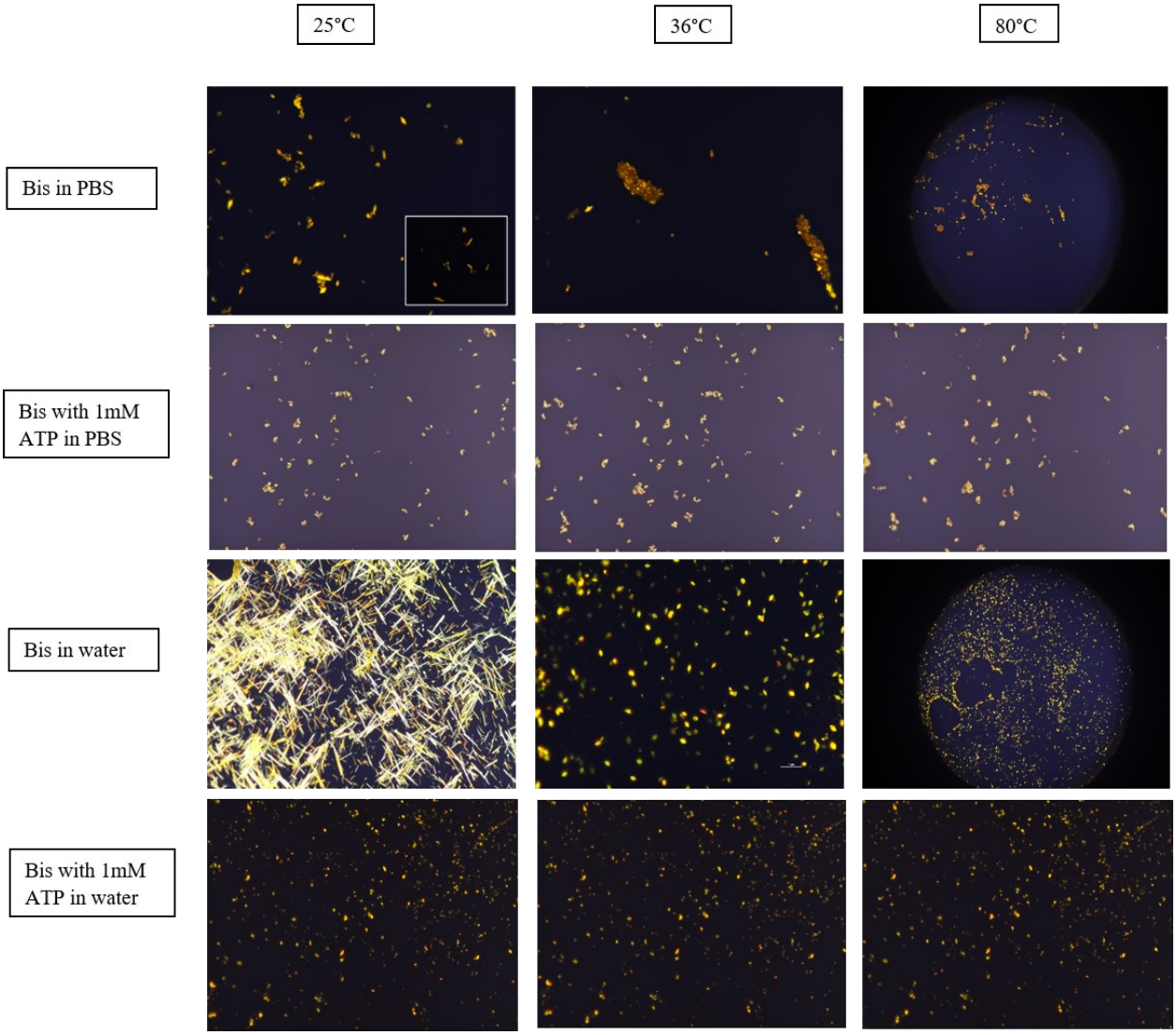

Figure 5 (Top left to bottom right panel): 2 μM bisantrene crystal solution in water and PBS under polarisation microscope. Image of bisantrene in TRIS:DMSO buffer is presented as inset in the first image. Sharp needle-like crystals along with diamond-shaped small crystals of bisantrene were observed in water (middle panel: left to right). No change in aggregation or crystallization was recorded with an increase in temperature when ATP was added to bisantrene in water (bottom panel: left to right) and PBS (middle panel: left to right). We also recorded polarisation images after cooling temperatures from 80˚C to 25˚C (data not presented). All images were taken after reaching respective temperatures at t_0.

Fluorescence of bisantrene crystals

Fluorescence intensity measurements were performed using time trace analysis on an FCS setup using SymphoTime software (see "Experimental section" in SI for details). A titration study was performed using different salt concentrations in various buffers, including water, PBS, and TRIS:DMSO. (Studies were also conducted to understand the specificity of bisantrene salt interaction with ATP salts, including ATP-Magnesium. No interaction could be detected in the TCSPC assessment in PBS buffer (Data not presented).

ATP concentrations were kept constant, and varying bisantrene salt concentrations were added to the PBS buffer to measure its interaction based on fluorescence intensity fluctuations. No significant fluorescence fluctuations were recorded when ATP was added to bisantrene salt. The highest counts/second were recorded for bisantrene in PBS without any ATP in the solution when excited at 485nm wavelength (laser with a long pass filter of 488nm).

TCSPC results:

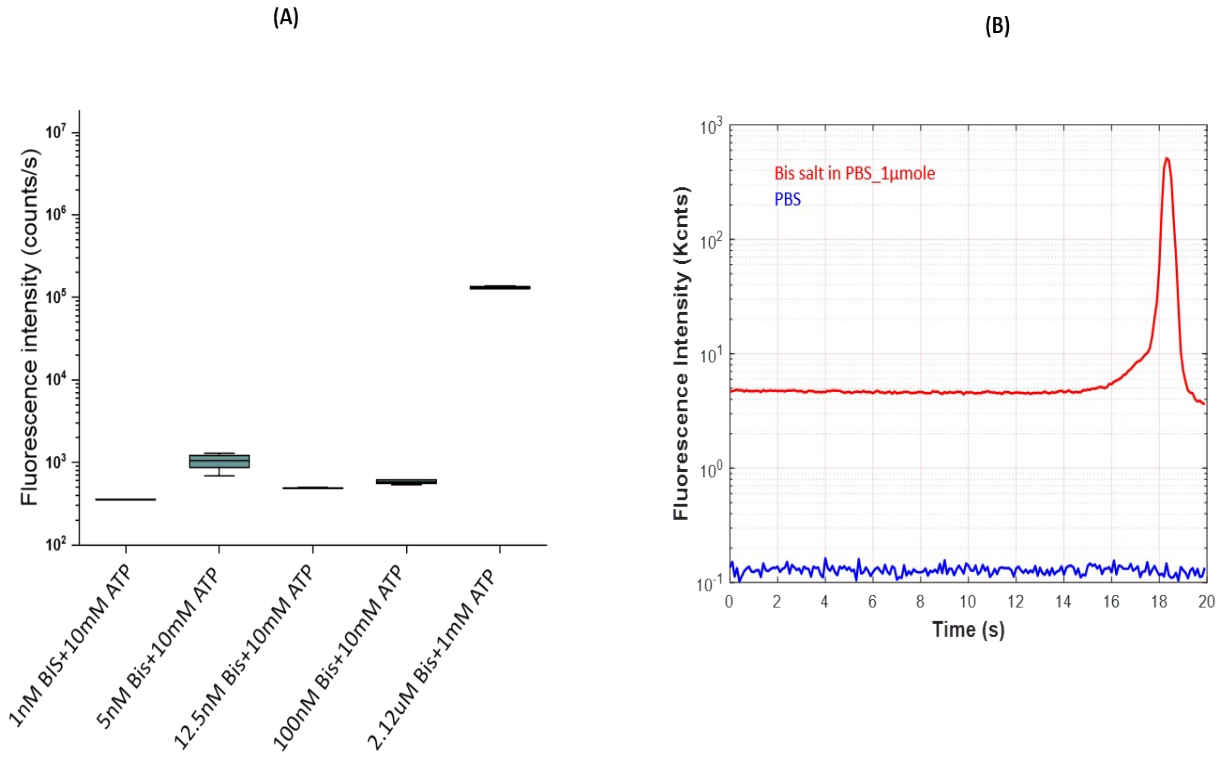

Figure 6: Time-correlated single photon counting analysis of bisantrene. (A) The fluorescence intensity of bisantrene with different ATP concentrations in PBS was measured with a laser filter λ_{ex} at 488nm. (B) The baseline fluorescence signal for PBS and bisantrene was measured at λ_{ex} at 488nm. Similar measurements were taken by titrating bisantrene and ATP concentrations, respectively. A single intense peak was observed at 16s from a large, bright aggregate/crystal. Experiments were performed in PBS buffer at 36˚C degrees on 35mm glass-bottom slides. These results are averaged from four individual experiments; each measurement averaged from five readings per experiment per sample concentration.

41 **Bisantrene crystal size**

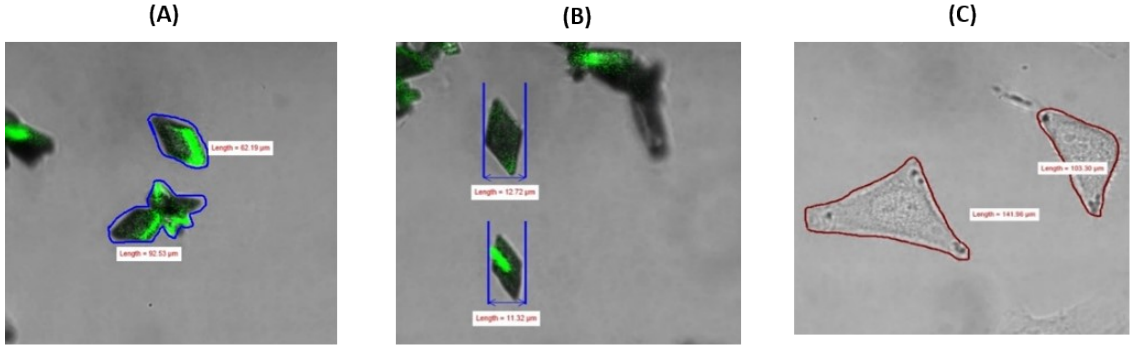

Figure 7: Area and length of 2 µM bisantrene crystals was measured. (A) Bisantrene crystal length in µm. (B) Bisantrene crystal width in µm. (C) Area of HeLa GFP cells seeded on 35mm glass bottom slide was measured.

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Bis-ATP interaction in buffer

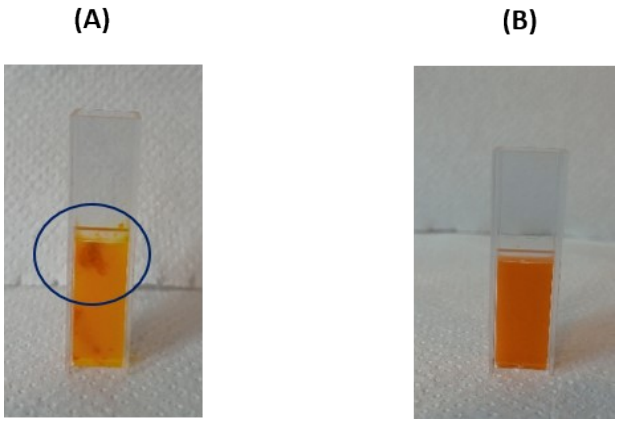

Fig 8: High concentration of bisantrene salt in different buffer conditions at 25˚C. (A) 10 µM bisantrene with 10 mM ATP in PBS. (B) 10 µM bisantrene with 10 mM ATP in TRIS:DMSO. Huge precipitates form in PBS buffer compared to those in TRIS:DMSO buffer when a higher concentration of bisantrene and ATP are added in buffer solutions.

Cytotoxicity of bisantrene

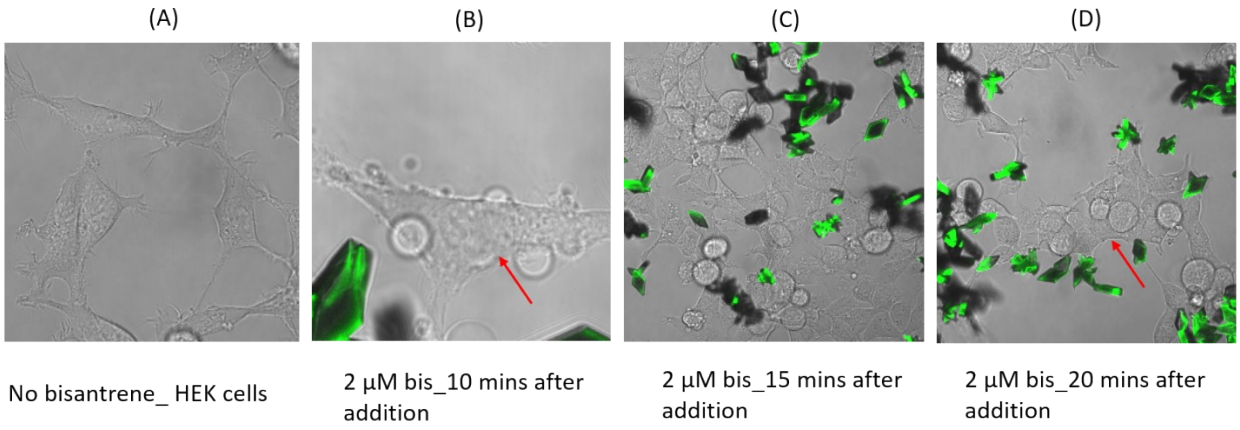

Figure 9: Cytotoxicity of bisantrene drug on HEK cells. (A) HEK cells in PBS before bisantrene addition at 40X. (B) Apoptotic bodies appear (red arrow) after 10 mins of drug addition; 60X. (C) and (D) Clumping and detachment of cells to become circular in shape (red arrows), 20 mins after drug addition at 20X.

Experimental section

Spectrophotometry: UV-VIS 2600 series spectrophotometer (model: A12595900701; version: 1.1) was used to perform spectrophotometer measurements with parameters range set between 300-700nm. Data obtained was analyzed using LabSolutions UV-Vis software.

Polarization microscopy: Nikon eclipse E400 microscope fitted with LWD 0.65 darkfield polarizing oblique microscope condenser was used to perform polarization studies. Linkam CI 94 was used for precise temperature control of Linkam MDS 600 and LNP heating/freezing stages to perform temperature cycles for all polarization experiments.

Bisantrene salt and Cells culture: Bisantrene dihydrochloride (sigma Aldrich; B4563) was used in all related experiments. From powdered bisantrene (471.39 g/mol), working solutions of 2 µM in 1ml PBS/TRIS:DMSO solvents were prepared and used during UV-VIS spectroscopy, confocal, and polarisation studies. Different concentrations of bisantrene salt were prepared (µM to nM) for supplementary TCSPC and polarisation studies. From 100 mM aqueous unconjugated ATP stock solutions, further dilutions were made (Thermofisher; R0441). HEK cells were cultured in DMEM media (Sigma-Aldrich) supplemented with 10% Fetal bovine serum (Sigma), 1% solution of penicillin (100 mg/ml), streptomycin (100 mg/ml) and 1% 2 mM of L-Glutamine (Sigma-Aldrich). Cells were maintained at 5% CO2 in an incubator (Biogenet) at 37˚C under humidified filter conditions. Cells were grown in T-75 corning flasks (Sarstedt AG & Co.) till passage 5 before seeding them on 35mm glass bottom cell culture dish (GBO) for confocal imaging.

Confocal Microscopy: Images were collected on Nikon A1 confocal setup. Melle's griot 488nm ion laser; model 35-IMA-410-019 was used for imaging, coupled with a FITC filter. t_0 measurements of cells were recorded in PBS containing calcium and magnesium ions.

Time-correlated single photon counting: TCSPC measurements were performed using a commercially available setup based on a confocal microscope (Nikon Eclipse TE2000U) coupled with Pico Harp 300 TCSPC module (PicoQuant). A climate chamber (Okolab) was used to perform all cell measurements providing temperature control (36±0.5°C) and atmosphere of required composition and humidity. A pulse diode laser (PicoQuant LDH-D-C-485; 485nm) driven by Sepia II module (PicoQuant) with a long pass filter of 488nm in the detector was used. The power of the laser was kept in the range of 5-10 µW. Molecules were observed and measured through 60×(N.A. 1.2) objective with water immersion.

Data acquisition and statistical analysis: Data was acquired from different concentrations of bisantrene with 5 measurements, lasting for 30 seconds each. Time correlated single photon counting denotes the number of photons counts/sec, plotted in Fig 6, SI.

Image and data plotting: TCSPC and UV-VIS spectrophotometric measurements were plotted on graphs using MATLAB (MathWorks's software).