# Targeted Photoresponsive TiO<sub>2</sub>-Coumarin nanoconjugate for efficient Combination therapy in MDA-MB-231 breast cancer cells: Synergic effect of Photodynamic Therapy (PDT) and Anticancer drug Chlorambucil

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#### 1. General information

Materials: All reagents were purchased from Sigma Aldrich and used without further purification. Acetonitrile and dichloromethane were distilled from CaH<sub>2</sub> before use. <sup>1</sup>H NMR spectra were recorded on a BRUKER-AC 200 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz).  $^{13}$ C NMR (50 MHz) spectra were recorded on a BRUKER-AC 200 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 77.0 ppm). UV/vis absorption spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer, fluorescence emission spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer, FT-IR spectra were recorded on a Perkin Elmer RXI spectrometer and HRMS spectra were recorded on a JEOL-AccuTOF JMS-T100L mass spectrometer. The morphology of NPs was measured through field emission scanning electron microscopy (SEM) with energy-dispersive X-ray (EDX) analysis (Hitachi S-4800) and transmission electron microscopy (TEM, JEOL, JEM-1200EX). Transmission Electron Microscopy (TEM) was measured on a FEI Tecnai G220S-Twin at 200 kV. The TEM sample was prepared by dispersing compounds in acetone and dropping on the surface of a copper grid. Photolysis of all the ester conjugates were carried out using 125 W medium pressure Hg lamp supplied by SAIC (India). Chromatographic purification was done with 60-120 mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) were used. RP-HPLC was taken using mobile phase acetonitrile, at a flow rate of 1mL / min (detection: UV 254 nm). Cell culture media and all the other materials required for culturing were obtained from Gibco, USA.

# 2. Characterization of DBMC-Cmbl Conjugate



**Figure S1:** <sup>1</sup>H NMR spectrum of Coumarin-chlorambucil conjugates (DBMC-Cmbl)



Figure S2: <sup>13</sup>C NMR spectrum of Coumarin-chlorambucil conjugates (DBMC-Cmbl)

# 3. Characterization of Ti- DBMC-Cmbl NPs

# (i) FT-IR overlaid spectra



Figure S3 : FT-IR overlaid spectra of TiO<sub>2</sub>, Ti-DBMC-Cmbl and free DBMC-Cmbl

(ii) Solid State UV spectra of Ti-DBMC-Cmbl NPs



Figure S4: Solid State UV spectra of Ti-DBMC-Cmbl NPs

# (iii) TGA of Ti-DBMC-Cmbl nanoparticles



Figure S5: TGA curve of Ti-DBMC-Cmbl nanoparticles under O<sub>2</sub> environment

### 3. 1. Photophysical studies of the NPs

#### (i) Measurement of fluorescence quantum yields<sup>1</sup>

The quantum yields of Ti-DMC and Ti-DBMC-Cmbl were determined by reference point method.<sup>1</sup> Quinine sulfate in 0.1 M  $H_2SO_4$  (literature quantum yield: 0.54) was used as a standard sample to calculate the QY of Ti-DMC and Ti-DBMC-Cmbl, which were dissolved in ultra pure water. The absorbance values of the solutions at the excitation wavelength were measured with UV–Vis spectrophotometer. Photoluminescence (PL) emission spectra of all the sample solutions were recorded by Hitachi F-7000 fluorescence spectrophotometer at an excitation wavelength of 360 nm.

$$\left[\frac{\phi_{\rm s}}{\phi_{\rm R}} = \frac{A_{\rm s}}{A_{\rm R}} \frac{({\rm Abs})_{\rm R}}{({\rm Abs})_{\rm s}} \frac{\eta_{\rm s}^2}{\eta_{\rm R}^2}\right]$$

Where  $\Phi$  represents quantum yield, Abs represents absorbance, A represents area under the fluorescence curve, and  $\eta$  is refractive index of the medium. The subscripts S and R denote the corresponding parameters for the sample and reference, respectively.

Table S1: Quantum yield o	f the fluorescent Ti-DMC and Ti-DBMC-Cmbl
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Sample	Intergrated emission intensity (I)	Abs. At 360 nm (A)	Refractive index of solvent (η)	Quantum yield at 360nm (Q)
Quinine sulfate	55337.365	0.0717	1.33	0.54 (known)
Ti-DMC	48628.257	0.545	1	0.062
Ti-DBMC-Cmbl	50036.057	0.555	1	0.0642

Fluorescence quantum yield (excitation wavelength 360 nm, error limit within  $\pm$  5%).

#### (ii) Loading of DBMC-Cmbl on TiO<sub>2</sub> nanoparticle by absorption spectra



**Figure S6:** (a) Standard UV-vis absorption spectra of DBMC-Cmbl (b) calibration curve for the concentration of DBMC-Cmbl

The concentration remain in the reaction mixture was calculated by the absorption spectra =  $1.75 \times 10^{-5}$ 

Initial concentration of the reaction mixture =  $1.46 \times 10^{-4}$ 

DBMC-Cmbl loaded on TiO<sub>2</sub>

= initial conc of DBMC-Cmbl - final conc of DBMC-Cmbl in reaction

medium

=  $1.46 \times 10^{-4} - 1.75 \times 10^{-5} \text{mol/mL}$ =  $1.2850 \times 10^{-4} \text{ mol/mL} \times \text{M.wt of DBMC-Cmbl}$ =  $0.06336 \text{ g of DBMC-Cmbl in 100 mg of TiO}_2$ =  $6.3360 \times 10^{-5} \text{ g in 1 mg of TiO}_2$ ~  $63.36 \mu \text{g/mg of DBMC-Cmbl loaded on TiO}_2$ .

### (iii) Fluorescence lifetime measurement<sup>2</sup>

To identify the excited state properties of the Ti-DBMC-Cmbl NPs, we carried out fluorescence lifetime measurements by the method of Time Correlated Single-Photon counting (TCSPC) using a HORIBA Jobin Yvon Fluorocube-01-NL fluorescence lifetime spectrometer. The sample was excited using a nano-LED at 340 nm and the signals were collected at the magic angle of 54.7° to eliminate any considerable contribution from fluorescence anisotropy decay.<sup>2</sup> The typical time resolution of our experimental set-up is ~ 800 ps. The decays were deconvoluted using DAS-6 decay analysis software. The acceptability of the fits was judged by  $\chi^2$  criteria and visual inspection of the residuals of the

fitted function to the data. Mean (average) fluorescence lifetimes were calculated using the following equation  $(2)^2$ :

$$\tau_{av} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$$
(2)

in which  $\alpha_i$  is the pre-exponential factor corresponding to the  $i^{th}$  decay time constant,  $\tau_i$ .

## (iv)Triplet State lifetime measurement <sup>3</sup>

A nanosecond flash photolysis set-up (Applied Photophysics) containing an Nd:YAG (Lab series, Model Lab 150, Spectra Physics) laser was used for the measurement of transient absorption spectra. The sample was excited at 355 nm (FWHM=8 ns) laser light. Transients were monitored through absorption of light from a pulsed xenon lamp (250 W). The photomultiplier (1P28) output was fed into a Tektronix oscilloscope (TDS 3054B, 500 MHz, 5 Gs s<sup>-1</sup>) and the data were transferred to a computer using TEKVISA software. The software ORIGIN 7.5 was used for curve fitting. The solid curves have been obtained by connecting the points using B-spline option. The samples were de-aerated by passing pure argon gas for 20 min prior to each experiment. No degradation of the samples was observed during the experiment.

#### 4.1 Synthesis and Characterization of Ti-DMC NPs

#### (a) Synthesis of Ti-DMC NPs

DMC (**3'**) was synthesized following classical Pechmann condensation<sup>4</sup> (**Scheme S1**) using pyrogallol and ethylacetoacetate as starting materials yielding white solid in 90% yield. 10 mL of  $10^{-4}$  (M) solution of compound **3'** in dry ethanol was prepared. To it 2 equiv. of KOH pellets were added. Further, 100 mg of prepared TiO<sub>2</sub> NPs were added under inert and dark condition with vigorous stirring. To monitor the reaction 0.1 mL aliquots were collected at every 15 min interval and their corresponding UV/vis spectra were recorded. This procedure was carried out up to 3 h. After completion of the reaction, the reaction mixture was diluted by 3 mL of ethanol and finally particles were collected by centrifuging the diluted reaction mixture. The resultant Ti-DMC NPs (4') were characterized by IR, UV/vis, fluorescence spectra, DLS, TEM images and solid state UV/vis spectroscopy.



### (b) Characterization of Ti-DMC NPs

### (i) FT-IR Overlay spectra of Ti-DMC



Figure S7: FT-IR Overlaid spectra of TiO<sub>2</sub>, Ti-DMC and DMC

(ii) UV/vis absorption and fluorescence emission spectra of Ti-DMC



Figure S8: Absorption and emission spectra of (a) DMC, (b) Ti-DMC

(iii) DLS and TEM images



Figure S9: (a) DLS, and (b) TEM images of Ti-DMC NPs

(iv) Solid State UV spectra



Figure S10: Solid State UV spectra of Ti-DMC NPs

# 4.2 Photoinduced Anticancer Drug release by Ti-DBMC-Cmbl nanoparticles

# Determination of photochemical quantum yield of Ti-DBMC-Cmbl $(\Phi p)^5$

These experiments were carried out using a previously described method. 1 mg of Ti-DBMC-Cmbl was dissolved in 1 mL of methanol / water (1:1 v/v) mixture in quartz cuvette. It was then irradiated under UV light by 125 W medium pressure Hg vapor lamp using a suitable filter 10% NaNO<sub>2</sub> in water, (the transmittance for the above filter = 410 to 600 nm). At regular interval of time, 20  $\mu$ L of the aliquots was taken and analyzed by RP-HPLC using mobile phase methanol, at a flow rate of 1 mL/min (detection: UV 254 nm). Peak areas were determined by RP-HPLC, which indicated gradual decrease of the caged compound with time, and the average of three runs. The reaction was followed until the consumption of the caged compound is less than 5% of the initial area. Based on HPLC data for each caged compounds, we plotted normalized [A] (HPLC peak area) versus irradiation time.We observed an exponential correlation for the disappearance of the caged compounds, which suggested a first order reaction. Further, the quantum yield for the photolysis of caged compounds was calculated using below equation.

$$\left[ \mathbf{\phi}_{\mathbf{p}} = \frac{\left( \mathbf{k}_{\mathbf{p}} \right)_{\mathbf{CP}}}{I_0 \left( \mathbf{F}_{\mathbf{CP}} \right)} \right]$$

where, the subscript 'CP' denotes caged compound.  $\Phi p$  is the photolysis quantum yield, kp is the photolysis rate constant, and  $I_0$  is the incident photon flux and F is the fraction of light absorbed. Potassium ferrioxalate was used as an actinometer.

Table S2 : Photochemical Quantum Yield of Ti-DBMC-Cmbl

Cage compound	Quantum yield (Φp)
Ti-DBMC-Cmbl	0.034

Photochemical quantum yield (error limit within  $\pm$  5%).



Figure S11: RP HPLC profile of photolysis of Ti-DBMC-Cmbl at different time intervals5. Evidence for Combination therapy by Ti-FADBMC-Cmbl nanoparticle

## 5.1. Synthesis and Characterization of Ti-FA-DBMC-Cmbl NPs

## (a) Synthesis of Ti-FA-DBMC-Cmbl nanoparticles<sup>6</sup>

Folic acid was loaded on Ti-DBMC-Cmbl nanoparticle surface following a previously reported procedure. <sup>6</sup> 0.300g of folic acid (Sigma) was dissolved in 0.1M sodium hydrogen carbonate (NaHCO<sub>3</sub>) solution adjusted to pH 5.5 with HCl and NaOH. Ti-DBMC-Cmbl nanoparticles were dispersed in deionized water to a concentration of 0.1 g/ml by sonication for 10min. The Ti-DBMC-Cmbl dispersion was then added slowly to the prepared folic acid solution with a volume ratio of 1:9, and the resultant mixture was stirred for one day (Scheme S2). The reaction mixture was finally dialyzed against 1mM NaHCO<sub>3</sub> solution for one day to remove unreacted folic acid, yielding folic acid-modified Ti-DBMC-Cmbl particles. Throughout the whole process for the preparation and preservation of folic acid modified Ti-DBMC-Cmbl dark and inert conditions were maintained.Thus formed Ti-FA-DBMC-Cmbl nanoparticles were further characterized by UV/vis, fluorescence spectra, FT-IR, TEM, DLS, Zeta potential and TGA measurements.



Scheme-S2: Synthesis of Ti-FA-DBMC-Cmbl nanoparticles

# (b) Characterization of Ti-FA-DBMC-Cmbl nanoparticles

(i) FT-IR spectral analysis for Ti-FA-DBMC-Cmbl<sup>6</sup>



Figure S12 : FTIR overlay spectra of Ti-FA-DBMC-Cmbl, Ti-DBMC-Cmbl and TiO<sub>2</sub>
(ii) UV/vis absorption and fluorescence emission spectra of Ti-FA-DBMC-Cmbl NPs



Figure S13: Normalised absorption-emission spectra of Ti-FA- DBMC-Cmbl



(iii) TEM and Dynamic Light Scattering (DLS) studies

**Figure S14:** TEM image (a) and Dynamic light scattering (DLS) spectra (b) of Ti-FA-DBMC-Cmbl nanoparticles

# (iv) TGA data of Ti-FA-DBMC-Cmbl nanoparticles



Figure S15: TGA-DTA curve of Ti-FA-DBMC-Cmbl

# (v) Solid State UV spectra of Ti-FA-DBMC-Cmbl NPs



Figure S16: Solid State UV spectra of Ti-FA-DBMC-Cmbl NPs 5.2 *in vitro* cytotoxicity measurements with Ti-FA-DBMC-Cmbl NPs



**Figure S17** : Cell cycle analysis by FACS calibur for 12h after treatment to UV light for 60 min.(a)Control, (b)Ti-DBMC-Cmbl, (c) Ti-FA-DBMC-Cmbl. Percentage of apoptotic cells are indicated as the proportion of cells that contained sub-G1 phase. Symbols M1, M2, M3, and M4 represented sub-G0/G1 peak, G0/G1 phase, S phase and G2/M phase, respectively. Each histogram plot is representative of three experiments

**Table S3**: Flow cytometric analysis of cell cycle distribution in MDA-MB-231 cells treated with PBS (control) and Ti-DBMC-Cmbl and Ti-FA-DBMC-Cmbl nanoparticles after treatment with UV ( $\geq$ 410nm) for 60min <sup>a</sup> Values are represented as mean  $\pm$  SD (n = 3)

	M1(Sub G0-G1) <sup>a</sup>	M2(G0-G1) <sup>a</sup>	M3(S) <sup>a</sup>	M4(G2-M) <sup>a</sup>
Control	1.73 ±0.01	95.21 ±0.03	$0.67 \pm 0.01$	2.46 ±0.005
Ti-DBMC-Cmbl	41.46 ±0.03	44.40 ±0.01	8.95 ±0.03	4.33 ±0.03
Ti-FA-DBMC-Cmbl	22.74 ±0.02	65.72 ±0.1	$6.01 \pm 0.02$	$0.34 \pm 0.01$

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