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Assessment of Dihydropyrimidinone based Nanocomposites as Multifunctional Anti-cancer Drug

Thangamani S., a† Hema Priya Mahendran, b, c† Rama Ranjan Bhattacharjee d, Sankarganesh Jeyaraj b, c*, and Kallol Mohanta a*

a PSG Institute of Advanced Studies, Coimbatore, Tamil Nadu 641 004, India.
b PSG Center for Molecular Medicine and Therapeutics, PSG Institute of Medical Sciences & Research (Affiliated to the Tamil Nadu Dr. MGR Medical University), Coimbatore, Tamil Nadu 641004, India.
c PSG Center for Genetics and Molecular Biology, Off Avinashi Road, Coimbatore 641004, India.
d Amity Institute of Nanotechnology, Amity University, Kolkata, West Bengal 700135, India.

† The authors have equal contribution.

* Email: (KM) kmohanta@gmail.com / kma@psgias.ac.in
   (SJ) sankar.jeyaraj@gmail.com

Abstract

Dihydropyrimidinones, a Biginelli compound, has been found to be a tumor inhibitor in last decade. The novel carbon quantum dot-dihydropyrimidinone (CQD-DHPM) nanocomposites have been prepared by a simple conjugation process by varying the ratio of DHPM to CQD. This conjugation leads fluorescent property to medicinal DHPM compounds and offers multifunctionality. The CQD-DHPM nanocomposites have been tested for anti-cancer activity against human non-small cell lung cancer A549 cell lines. It has been found that conjugating CQD with DHPM also improves the anti-cancer activity and reduces cytotoxicity for healthy cells. The fluorescent property of the composites also enables them to serve as bioimaging fluorophore.

Keywords: DHPM; CQD; nanocomposite; anti-cancer; fluorophore; bioimaging
Introduction

Efficiently processable dihydropyrimidinones (DHPMs) are well known for their strong pharmacological properties such as antioxidant, antimicrobial, antitumoral and inhibitor to HIV-1, lipoxygenase etc. They are also proved to demonstrate their ability against HepG2 and HeLa cell lines, human polyomavirus (HPyV), Hsp90 C-terminal inhibitors. DHPM compounds specifically inhibit Eg5 motor spindle protein and restrict mitotic progression of cells and hence can be used as anti-cancer drug. Matos et al. has reported that DHPM and its derivatives were tested on various cancer cell lines representing lung, breast, prostate, colorectal cancer etc. The half maximal inhibitory concentration (IC50) is a measure of inhibitor potency to inhibit certain biological function; said that, lower IC50 value of a drug to inhibit tumor cell-growth is more recommendable since it would have less side-effects. Ragab et al. has reported that the U251 (Glioblastoma multiforme) and OVCAR-03 (Ovarian cancer) were found to be more sensitive to DHPM derivatives which affected the cell growth at IC50 <10µg/mL. Non-small cell lung carcinoma (NSCLC) is a devastating cancer arising in the lung epithelial cells. NSCLC accounts for about 85% of lung cancer. It usually grows and spreads slowly compared to small cell lung cancer and is considered as the common lung carcinoma. This cancer remains aggressive with increasing incidence and poor survival rate despite of current multimodal treatment. NSCLC has been modeled on A549 cell line which is adenocarcinomic and composed of human alveolar basal epithelial cells. In a previous study, the cytotoxicity against A549 cell lines were tested for compounds like 2-(5-nitrofurfurylidene)-5-(aryl)-7-(2,4-dichloro-5-fluorophenyl)-5H-thiazolo[2,3-b]pyrimidin-3-ones, 2-(5-arylfurfurylidene)-5-(aryl)-(2,4-dichloro-5-fluorophenyl)-5H-thiazolo[2,3-b]pyrimidin-3-ones, and 4-aryl-6-(2,4-dichloro-5-fluorophenyl)-3,4-dihydropyrimidin-2(1H)-thiones which exhibited a minimum IC50 of 16.5 µM.
In modern drug innovation, it is carefully contemplated to include multifunctionality to a drug so that one single composition would be able to address several purposes related to the ailment. One of the key aspects of the multifunctional drug is to track the drug within body or in body-fluids so that the dosage of the drug and the monitoring of drug to the targets as well can be optimized. Fluorescent tagging of a drug molecule enables real-time monitoring of the drugs inside the living systems. Fluorescence properties aids to interrogate drug-target interactions, drug-tubulin interfacing, pharmaco-dynamic consequences.\textsuperscript{14} Since DHPM compounds do not inherit any fluorescence property;\textsuperscript{15} luminous hybrid DHPM composites for applications like drug detection and bioimaging are limited. With the advent of nanomaterials, targeting the ailing cells and tracking drugs getting simpler. Attaching/ conjugating a fluorescent nanomaterial to the particular drug molecule to achieve fluorescent properties is a common practice in current research.\textsuperscript{16} CQDs are the newest class of carbon nanomaterials and it has various advantages like simple and inexpensive synthesis, tunable properties, modifiability etc.\textsuperscript{17} CQDs have dynamic fluorescence property, high chemical stability, low cytotoxicity and biocompatibility.\textsuperscript{18, 19} Their biocompatible features combining with fluorescent properties enable them to efficiently serve as effective fluorophore for bioimaging and probe for drug delivery.\textsuperscript{20} Wang \textit{et al.} reported that the CQDs modified with zinc oxide and silica-based materials have biological applications for their fluorescence properties.\textsuperscript{21} Prasad \textit{et al.} have shown significant anti-cancer property of CQDs made from small organic molecules against breast cancer cell line.\textsuperscript{22} But reports on the integration of CQDs with any pharmacological molecules are rare – until Qu \textit{et al.} synthesized CQD-TPEA.\textsuperscript{23} This nanocomposite was prepared by attaching TPEA (N-(2- amino ethyl)-N,N,N-tris (pyridin-2-ylmethyl) ethane-1,2-diamine) with water-soluble CQD particles. TPEA-CQD composite has shown low cytotoxicity and has been used for intercellular sensing and bio-tagging of Cu\textsuperscript{+2}.\textsuperscript{23}
The aim of this study is to prepare a hybrid fluorescent monastrol composite based on the combination of DHPM and CQD i.e. CQD-DHPM, to evaluate the anti-cancer property in A549 cell line and determining its bioimaging capability. The surface of the CQDs contains suitable functional group (polystyrene sulphonate) as capping agent so that the optical properties of the CQDs and the therapeutic performance of DHPM could be retained in the new conjugation. The pharmacological agent, CQD-DHPM nanocomposite developed in this study, has been evaluated to estimate its anti-cancer activity against A549 cell lines. At this end, we evaluated the effect of our novel CQD-DHPM nanocomposites at different ratios in combination and as individuals against A549 cell lines as a model for NSCLC. It was found that making the nanocomposites in fact increases the reactivity of the DHPM compound (without subgroup) as well as lowers the cytotoxicity for the healthy cells.

**Experimental Section**

**Materials**

Benzyl alcohol, hydrogen peroxide, ethyl acetoacetate, urea and citric acid (CA) were purchased from LOBA Chemie and were used without purification. Jeffamine® was obtained as a gift sample from Huntsman India Ltd. Phosphotungstic acid (PTA) and poly (sodium 4-styrene sulfonate) (PSS) were purchased from Fluka and Sigma-Aldrich respectively and used as received. The standards and the reaction mixtures were spotted on TLC Silica gel 60 F254 purchased from Merck Chemicals. Milli-Q water was used in all preparation.

Peripheral blood mononuclear cells (PBMCs) from healthy controls, Human Non-small cell lung cancer cell, A549 cell line for pharmacological tests has been used with proper ethical guideline
from Institutional Human Ethics Committee, PSG Institute of Medical Sciences and Research, Coimbatore.

**Synthesis of Dihydropyrimidinone and Carbon Quantum Dots**

Dihydropyrimidinones molecule is prepared by Biginelli reaction. Synthesis of the Dihydropyrimidinone is followed route as described elsewhere. Carbon Quantum Dots also prepared as given in our previous report. Detailed procedures for Dihydropyrimidinone and CQD synthesis are given in the Supplementary Information.

**Preparation of CQD-DHPM nanocomposites**

The CQD-DHPM nanocomposites preparation of different proportions of CQD to DHPM was carried out following; 10 mg of CQD in 10 ml of water was stirred for the CQD dispersion, 10, 20, 30 & 40 mg of DHPM were completely dissolved in 10 ml of ethanol each (for 1:1, 1:2, 1:3 and 1:4 ratios respectively) and stirred continuously. Then DHPM solution was added drop wise to the CQD solution with continuous stirring. The immediate sample of the CQD-DHPM was taken for analysis. Samples were collected at regular intervals of 15 minutes till 90 minutes. The solution was initially characterized by fluorescence microscopy. Formation of nanocomposite was confirmed by optical, compositional, thermal and morphological analysis.

**Characterization tools**

Ultraviolet-Visible absorption (UV-Vis) spectra were recorded with Shimadzu UV-1800 spectrophotometer in the range of 200-800 nm in a 3 mm path-length cuvette with all the samples at 1 mg/ml in DI water. Fourier Transformed Infra-Red absorption (FT-IR) spectra were recorded using Shimadzu IR Affinity series 1S in the range of 4500-600 cm⁻¹and Nuclear Magnetic Resonance (NMR) spectra were recorded by Bruker BioSpin GmbH. Thermo-Gravimetric Analysis measurements were carried out by NETSCH STA 449F3 Jupiter thermal analyzer under
nitrogen atmosphere. Fluorescence emission and excitation spectrum was recorded with Shimadzu RF-5301PC Spectrofluorophotometer of 1 mg/ml concentration of samples in aqueous media. The fluorescence microscopic studies were carried out using Nikon Upright Microscope Eclipse Ni-U. The samples were analyzed under different excitation wavelengths in blue (Ex-330-380 nm), green (Ex-450-490 nm) and red (Ex-510-560 nm) region. High Resolution Transmission Electron Microscope (HR-TEM) analysis was performed for nanoparticles measurement using JEOL JEM 2100 model.

**Cell culture:**

A549 cells were grown in complete DMEM, at an optimum temperature of 37 °C with 5% CO₂. Culture media was supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, 1% Antibiotic-antimycotic solution. The cells were grown aseptically in tissue culture flasks (T 25 cm²). Fresh PBMCs were also included to determine whether the compounds displayed any toxicity towards normal cells. The PBMCs were isolated by density gradient centrifugation using Ficoll-Paque overlay method, and grown aseptically in tissue culture flasks (T75²) using medium that comprised RPMI-1640, 10% heat inactivated filtered fetal bovine serum (FBS), 1% Streptomycin and Penicillin solution. The cultures were incubated in humidified 5% CO₂ incubator at 37 °C.

**Cytotoxicity assay:**

The *in vitro* cytotoxicity of CQD-DHPM nanocomposites using MTT assay was carried out in 96-well, flat bottomed microtiter plates. A volume of 200µL complete culture medium containing 5 × 10³ cells was seeded to each well on the first day of the experiment and incubated for 24 hrs for the cells to attach to the wells. The following day, old media was replaced with fresh media.
containing different concentrations (in µM) (25, 50, 100, 200, 300, 400 and 500) of the drug and incubated at an optimum temperature of 37 °C with 5% CO₂ for 24 hrs. To determine the cytotoxicity, 10-15µL of 5mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylerazolium bromide (MTT) was added followed by incubating the plates for 3 hours at 37 °C in the dark. After 3 hours, the media was aspirated and 100µL of DMSO was added to each well and absorbance was read at 590 nm by using Varioskan Flash multimode microtiter plate reader. IC50 values were determined by non-linear regression analysis. To determine the in vitro cytotoxicity of DHPM on freshly isolated PBMCs from healthy blood donors, around 1x10⁵ cells were seeded per well and incubated with different concentrations (in µM) (25, 50, 100, 200, 300, 400 and 500) of the compound at 37 °C with 5% CO₂ for 72 hrs to determine the maximum cytotoxic effect. After incubation, MTT assay was performed and absorbance was read at 590 nm. The mean percentage of post treatment viable cells was calculated using the following formulae.

\[
\%\text{Cell Viability} = \frac{\text{Absorbance of treated cells} \times 100}{\text{Absorbance of untreated cells}}
\]

\[
\%\text{Cytotoxicity} = 100 - \%\text{Cell Viability}
\]

**Fluorescence Microscopy:**

Cells from flasks were harvested by trypsinization and 0.5x10⁴ cells were seeded on a sterile 8-well chamber slide over night at 37 °C for fluorescent microscopy. The proceeding day, cells were treated with drug compounds (CQD & CQD-DHPM Nano-composites) at IC50 Concentration and incubated at 37 °C for 24 hrs. After incubation the media was aspirated and the attached cells were washed twice with PBS. The chamber walls were then carefully removed with forceps and the
cells were then covered with a cover slip and directly viewed under fluorescent microscope (using UV light). Images were taken after mounting the slides with a cover slips and mounting medium.

**Results and Discussion:**

In a previous study, Dihydropyrimidinone molecule has been evaluated for antitumor activity against A549 cell lines and the half maximal inhibitory concentration i.e. IC50 value was identified at 202.6µM. Although DHPM has commendable biological activities but it lacks fluorescent property, which restricts its usability in bioimaging. Fluorophore for the DHPM molecules is not readily available or existing materials are not suitable as fluorophore with DHPM molecules since it may alter the pharmacological activity of the drug molecules. Till now, mostly coumarin based materials were used as efficient fluorophores due to its benign nature, although coumarin has its own limitations like photo bleaching and photo damaging of the embodying tissues due to its low excitation and emission wavelengths. Currently developed carbon quantum dots (CQD) possess interesting fluorescent properties. It has a wide range of excitation dependent emission and also CQDs are non-cytotoxic. The carbon quantum dots are relatively inactive and can be functionalized according to the needs. These advantages make carbon quantum dots as one of the promising alternatives to coumarin as fluorophore.

The CQD-DHPM nanocomposite has been analyzed by the High-Resolution Transmission Electron Microscope (HR-TEM) to study the size distribution and lattice properties of the CQD-DHPM nanocomposite. Figure 1a&b are showing the CQD and CQD-DHPM (ratio 1:2) and their magnified version respectively. It can be seen from Figure 1a that individual CQD particles have mostly oblong shapes with a size of 5±2 nm. On the other hand, CQD-DHPM particles observed as cloudy and agglomeration of organic molecules with sparse tints of firm structures, Figure 1b. Due to the presence of strong interatomic interactions, molecules did not show a defined structure.
The deep black points within this cloudy formation could be the CQDs/ CQD clusters which are attached with the molecules. The sizes of these formations are about 200 nm which is way bigger than the single CQDs (~ 5 nm) and reveals the structure of the conjugate. The Atomic Force Microscope morphology study of the CQD-DHPM (ratio 1:2) also supports the fact has been seen in Transmission Electron Microscope. Figure S1a&b are the topographies at different magnification of CQD-DHPM particles scattered on a glass substrate. The AFM images show the presence of the ~ 0.2μm particles that represent CQD-DHPM particle and >10 nm CQDs can be observed in the background.

The thermogravimetric analysis (TGA) of the pure DHPM and CQD-DHPM (1:2 ratio) in nitrogen atmosphere was carried out to confirm the conjugation of DHPM molecules with CQDs. The results are shown in Figure 2. The TGA thermogram (Figure 2) shows the complete decomposition of DHPM takes place at 300°C as evidenced from the endothermic heat exchange in the DSC curve (not shown here). But for the CQD-DHPM the decomposition occurs at a lower temperature. This suggests that there is a conjugation between the inorganic CQDs and the organic DHPM – which triggers the decomposition of the organic part at lower temperature by lowering the bond strength. It could be seen that there was a gradual weight loss even before that, which may be due to slow burning of the surface functionalization material (PSS) of CQDs. Decomposition is incomplete since ~15% of retained which decays slowly. This should be the inorganic part which has robust C-C structure. However, it would be noted that phase transition temperature of the DHPM molecules did not change though the conjugation with CQDs strongly altering thermodynamic property of the DHPM molecules.

The conjugations between the DHPM molecules and the CQDs have been evidenced from the FTIR spectra analysis of the DHPM and CQD-DHPM samples. A detailed result has been given
in the Supplementary Information. Figure 3 shows the FTIR spectra of the samples and corresponding tables from S1 to S6 indicate the presence of O-H stretching (broad, ~3400 cm\(^{-1}\)) N-H stretching (broad, 3200 cm\(^{-1}\)), C=O stretching (1750 cm\(^{-1}\)), C-N stretch (~1400 cm\(^{-1}\)), C-O-C stretching (1226, 1296 cm\(^{-1}\)) and C-N-C in amines (1050 cm\(^{-1}\)). For the nanocomposites, presence of O-H stretching and systematic shift of C-N stretching towards lower wave numbers are noticeable which confirms the conjugation of CQDs and DHPM molecules and hints probable formation of C-N bonds between the two components of the composites.

The pure DHPM and CQD-DHPM nanocomposites were also analyzed by \(^1\)H NMR analysis and detailed spectral analysis of samples was mentioned in SI (Figure S2-S4 and Table S7-S11). The NMR spectra and their corresponding chemical shifts of the 1:2 ratio CQD-DHPM were given in Figure 4 and Table 1. The pure DHPM molecules observed that the NMR signals at 8.17 ppm and 7.26 ppm (m, 3H) were indicative of an aromatic and confirmed the pyrimidine aldehyde. The two hydroamides signals could be seen at 5.39 ppm and 5.86 ppm. The presence of 3.7 ppm (s, 6H, 2 CH3) observed was indicative of the dimethylamino moiety. While the peak of 2.3 ppm (s, 3H) shows the presence of an isolated methyl group and confirms the formation of DHPM. When the DHPM molecule was conjugated with CQD, the NMR spectra exhibit slight variations in peak positions as shown in the Figure 4. The CQD-DHPM nanocomposites were observed to have a peak at 2.2 ppm (m, 2H) for methyl on linear chain whereas the peaks from 3.00 ppm to 4.00 ppm were for isolated methyl group. The presence of 5.14 ppm peak indicates dimethylamino group. The aromatic protons in the composite can be discovered from the peak at 7.24 ppm (m, 3H). It should be noted that signals arising around 9 ppm to 7.32 ppm, where there should be a probable indication of singlet consisting of a single H-proton.
From the above discussion it is clear that DHPM and CQDs have been conjugated. Several research groups previously reported that carbon nanomaterials including CQDs possess oxygen containing functional group on their surface and they can be conjugated by groups like amines.\textsuperscript{15, 27} However, in our case, the conjugation might be happened by forming covalent bonds between amine groups of DHPM with the surface functionalization group on CQD. Now we would like to see whether this conjugation alters the optical properties of the CQDs. This is essential that CQDs retain their fluorescent characteristics even within the nanocomposites so that it can be used as an efficient fluorophore. The prepared nanocomposite samples have been analyzed by UV-Vis spectroscopy, fluorescence spectroscopy and fluorescence microscopy.

A prominent absorption peak at 284 nm is observed for 3, 4-dihydropyrimidinones (DHPM) along with a small hump around 221 nm (Figure S5). Both the absorption peak and hump for DHPM appear as a result of $n\rightarrow\pi^*$ transition of C=O; the presence N atom in the benzene ring of DHPM is strengthening the peak at 284 nm. On the other hand, CQDs have the absorption peak at 223 nm (Figure S5 & Table S12). This absorption peak arises from $\pi\rightarrow\pi^*$ transition of C=C present in CQDs. When DHPM is conjugated with CQDs, the 284 nm peak is slightly red shifted (~2/3 nm) in CQD-DHPM nanocomposites. This small shift may be attributed to the strains in the heteroatom ring of the DHPM molecules in the composite. These variations are due to strains in the heteroatom ring caused by the addition of functional chains to the ring as well as quantum dot.

Emission spectra of aqueous CQD suspension were recorded at various excitation wavelengths. It can be seen from Figure 5 that with increasing the excitation wavelength from 310 nm to 410 nm, the emission peak slowly shifts from 440 nm to 490 nm with varying intensities. However, the strongest emission peak of CQDs is appeared at 442nm for the excitation wavelength of 360 nm. These results are compatible with our previous reports.\textsuperscript{15} Emission spectra of different CQD-
DHPM nanocomposites as aqueous suspension have been shown in Figure 5. These spectra are similar to that of CQDs only. This evidence was observed that the optical property of the CQDs has remained almost unaltered in the nanocomposites since the DHPM have no fluorescence property. The only difference can be seen for the CQD-DHPM nanocomposites are that the change of the strongest emission peak position and the excitation wavelength for that highest emission peak. For the different CQD-DHPM composite both of the peaks have been red shifted by 10 nm. The excitation dependent emission feature of the CQDs enables the nanocomposites for multicolor labeling of cells in fluorescence imaging.

The crystals of dihydropyrimidinones and the nanocomposites are visible under microscopy. Interestingly, the result of the microscopy reveals that the fluorescence images of CQD-DHPM nanocomposite of different fluorescent structures when the proportions of dihydropyrimidinones to CQD have been varied. Figure 6 shows that different structures of CQD-DHPM conjugates of ratio 1:1, 1:2, 1:3 & 1:4 for the CQD:DHPM respectively. Although the exact reason for the formation of such different structures is not been yet explored, but probably with increasing DHPM molecules the availability of growth sites on CQDs surface has guided the directionality of crystal formation in different shape. The nanocomposite formation and their structural properties were confirmed by the above characterization techniques.

In the recent years, interests on dihydropyrimidinones and their analogues have increased vividly as anti-cancer drugs. In contrast to other anti-cancer drugs that perturb mitosis by binding to the protein tubulin like natural taxanes, vinca alkaloids and epothilones, monastrols (DHPM derivatives) specifically affects the cell division by a new mechanism. Kinesin Eg5, a spindle motor protein, is an attractive therapeutic target to prevent cell cycles by mitosis. Monastrol drugs inhibit this Kinesin Eg5 to form bipolar spindle by polar migration of centrosome resulting in
monoastral spindle formation and thus restricting mitosis.\textsuperscript{8, 28, 29} Several researchers for decades synthesized DHPM with modifications in their structures to enhance the activity against microbial pathogens and cancer cells of interest.

In this study, we used a) DHPM (dihydropyrimidinones) alone b) CQDs alone and c) CQD-DHPM (Carbon Quantum Dots-DHPM) nanocomposites at four different ratios: 1:1, 1:2, 1:3, 1:4 for evaluating the cytotoxicity activity against A549 cell lines. Analyzing the percentage of viability and percentage of growth inhibition of DHPM compounds, we observed a dose dependent cytotoxicity of all the drugs against A549 cell line. The percentage of viability decreased proportionally to higher drug concentrations as shown in Figure 7a. After 24 hrs of treatment, A549 cell viability was reduced to a greater extent at higher concentrations (i.e., above 200 µM) while using CQD-DHPM nanocomposites (1:1, 1:2, 1:3, 1:4). To establish the optimal treatment concentration for DHPM and its composites, IC\textsubscript{50} values were calculated and compared as shown in (Table 2). The half maximal inhibitory concentration of DHPM compounds against A549 cells were calculated, with an R\textsuperscript{2} value of 0.99.

We used healthy PBMCs to evaluate any cytotoxicity of the compounds against healthy host cells. There are earlier reports of anti-tumor monastrols where the cytotoxicity studies to normal cells have been withheld.\textsuperscript{30} The toxicity of our synthesized DHPM compounds against normal cells (PBMCs) remained low even at the half maximal inhibitory concentration (<20%) as shown in Figure 7b. In the research work published by Venugopal \textit{et al.}, the DHPM derivatives exhibited up to 20% growth inhibition at 50 µg/mL i.e. ~50 mM.\textsuperscript{31} The Lithium-Acetate-Mediated Biginelli compounds required more than 100 µM to exert 50% cytotoxic activity against A549 cell line. In this current study, PBMCs viability was found to remain more than 60% even after 72 hrs of CQD-DHPM treatment as shown in Figure 7b (note that the maximum concentration used for all the
drugs is 500 µM). We confirm CQD-DHPM analogues are non-toxic to PBMCs on comparing the activity of them to A549 cells (Table 3). The percentage of growth inhibition of the drugs against both the A549 cell line and PBMCs were compared at 100 µM concentration and shown in Table 3. The higher percentage of growth inhibition of cancer cells at lower dosages and for much less incubation time compared to the normal cells is a proof that the drugs contain the inhibition specificity towards the A549 cancer cells.

Comparing the DHPM derivatives treated against breast cancer cell lines model by Guido et al. using MCF7 and MDA-MB-231 cell lines,\textsuperscript{30} the novel synthesized DHPM molecule and CQD-DHPM nanocomposites in our study have proven to show significant decrease in cell viability gradually in all concentrations. CQD-DHPM nanocomposites (1:2) had the lowest half maximal inhibitory concentration when compared to other combinations. This behavior may be attributed to the number of accessible DHPM molecules in the combination along with structural advantages of the CQDs. However, CQD alone has also showed inhibitory activity at higher concentrations against A549 cells.\textsuperscript{22} In the research work published by Guido et al., the DHPM and its derivatives were used at concentrations up to 1 mM.\textsuperscript{6,30} The DHPM derivatives exhibited over 80% of cell growth inhibition with IC50 around 6 to 35 µM.\textsuperscript{31} It has been previously shown that monastrol mimic Biginelli DHPM derivatives exhibited cytotoxicity against HepG2 with half maximal inhibitory concentration at 120.62µg/mL and it exhibited weak toxicity towards HeLa cell lines with IC50 200 µg/mL.\textsuperscript{6}

The \textit{invitro} examination of DHPM in combination with CQD was viewed under fluorescence microscopy for tracking the location of nanocomposites inside the target cells. The microscopic image revealed the entry of CQD-DHPM nanocomposites into the cells exciting at 450 nm as
shown in Figure 8. The PL analysis (Figure 3b) also supports the excitation of the composites at
the same wavelength.

This phenomenon of successful fluorescence property of CQD-DHPM composites is corelating
with the report of M.J. Molaei et al.\textsuperscript{32} that carbon-based materials with a particle size larger than
70 nm can also emit fluorescence and can be used for bio-imaging application, although particles
lesser than 10 nm are commonly used for bioimaging applications.\textsuperscript{32} Further studies need to be
done in order to elucidate the molecular pathways in NSCLC cancer cell line death induced by
DHPM compound and its nanocomposites.

Conclusion

We have prepared nanocomposites of CQDs and DHPM molecules and confirmed their formation
by different characterizations. These newly synthesized CQD-DHPM nanocomposites in our study
were found to have moderate activity against A549 cancer cell lines at lower concentration. The
1:2 ratio of CQD-DHPM composites has exhibited robust cytotoxicity activity against NSCLC
compared to other ratios and DHPM alone. These composites are more specific to cancer cells and
exhibit very low toxic effect on normal cells (PBMCs). Due to superior fluorescent properties,
these composites show bioimaging capability for the excitation of 450 nm. Thus, a multifunctional
nanocomposite has been developed in this study which showed moderate anti-cancer activity
against NSCLC cell lines and used as a fluorophore.

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Supplementary Information

Supplementary Information of the article would be available from the journal website.

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### List of Tables

**Table 1:** Chemical shift values of pure DHPM (PTA-Jeffamine® pH-7.5) catalyzed reaction and CQD-DHPM (1:2 ratio).

| S.No. | DHPM Peak (ppm) | Chemical Shift (J) | CQD-DHPM (1:2) (ppm) | Chemical Shift (J) |
|-------|-----------------|--------------------|----------------------|--------------------|
| 1     | 1.14-1.17       | 3.08 (t, 3H) CH₃  | 1.09-1.12            | 1.00 (t, 3H) CH₃  |
| 2     | 1.23            | 1.55 (m, 6H)      | 2.25-2.51            | 0.98 (m, 2H, N-CH₂)|
| 3     | 1.46            | 0.59 (s, 3H) CH₃  | 3.35-4.01            | 0.66 (m, 2H) CH₂  |
| 4     | 1.78            | 3.32 m, 2H, CH₂   | 5.14                 | 0.33 (s, 1H) NH   |
| 5     | 2.34            | 3.05(s, 3H) CH₃   | 7.24-7.74            | 2.00(m, 3H) ArH   |
| 6     | 3.70-3.72       | 1.08(m, 2H, N-CH₂)| 9.20                 | 0.26 (s, 5H) Aromatic |
| 7     | 4.05-4.08       | 2.09 (m, 2H) CH₂ ethyl | - | - |
| 8     | 4.83            | 1.00m, 1H, C4-H   | -                    | - |
| 9     | 5.39            | 1.05 (s, 1H) NH   | -                    | - |
| 10    | 5.86            | 1.04 (s, 1H) NH   | -                    | - |
| 11    | 7.26-7.31       | 3.89(m, 3H) ArH   | -                    | - |
| 12    | 8.17            | 1.00 (s, 5H) Aromatic | - | - |
Table 2: Levels of cytotoxicity exhibited by the DHPM composites against A549 (Non-Small Cell Lung Carcinoma) 24 hrs treatment.

| S. No. | DHPM Composites                        | Cytotoxicity (IC50 in µM) |
|--------|----------------------------------------|--------------------------|
| 1      | DHPM (dihydropyrimidinones)            | 202.6                    |
| 2      | CQD (Carbon quantum dots)              | >350                     |
| 3      | CQD-DHPM nanocomposite (1:1)            | 160.4                    |
| 4      | CQD-DHPM nanocomposite (1:2)            | 128.5                    |
| 5      | CQD-DHPM nanocomposite (1:3)            | 232.5                    |
| 6      | CQD-DHPM nanocomposite (1:4)            | 181.6                    |

Table 3: Comparison of percentage of growth inhibition of DHPM composites against A549 and PBMCs at 100 µM concentration. A549 -Non-Small Cell Lung Carcinoma (NSCLC), PBMCs- Peripheral Blood Mononuclear Cells.

| S. No. | DHPM Composites                        | Percentage (%) of growth inhibition |
|--------|----------------------------------------|-------------------------------------|
|        |                                        | A549 (24 hrs) | PBMCs (72 hrs) |
| 1      | DHPM (dihydropyrimidinones)            | 42.6        | 26.42         |
| 2      | CQD (carbon quantum dots)              | 43.4        | 19.17         |
| 3      | CQD-DHPM nanocomposite (1:1)            | 46.5        | 32.79         |
| 4      | CQD-DHPM nanocomposite (1:2)            | 47.5        | 20.94         |
| 5      | CQD-DHPM nanocomposite (1:3)            | 44.3        | 35.9          |
| 6      | CQD-DHPM nano composite (1:4)           | 43.4        | 43.13         |
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Figure 1: HR-TEM analysis of a) pure CQD and b) CQD-DHPM nanocomposites 1:2 ratio.
Figure 2: TGA analysis of pure DHPM and CQD-DHPM nanocomposites 1:2 ratio.

Figure 3: a) and b) represents the FT-IR spectroscopy analysis for DHPM, CQD and CQD-DHPM spectral data.
Figure 4: 1H NMR analysis of a) pure DHPM and b) CQD-DHPM nanocomposites 1:2 ratio.
Figure 5: Photoluminescence spectra of a) CQD and b) CQD-DHPM nanocomposites 1:2 under different excitation and emission wavelengths (310nm-410nm). With change in excitation wavelength the emission peak wavelengths also change.
Figure 6: Fluorescence microscopy images of CQD-DHPM nanocomposites 1:1, 1:2, 1:3, and 1:4 ratios under blue, green and red filter.
Figure 7: Cytotoxicity effect of DHPM, CQD-DHPM against A549 and PBMCs. Dose dependent cytotoxicity of (a) DHPM, CQD & CQD-DHPM nanocomposites against A549 cell line after 24 hrs treatment and (b) CQD-DHPM nanocomposites against PBMCs after 72 hrs treatment. For all the cases, the “p” value is < 0.0001.
Figure 8: Fluorescence imaging showing CQD and CQD-DHPM entry into the target cells. *In vitro* examination of fluorescence microscopic analysis under excitation wavelength of 380 nm and bright field of a) A549 untreated cells b) CQD (350 µM) c) CQD-DHPM conjugates of ratio 1:1 (160.4 µM), d) 1:2 (128.5 µM), e) 1:4 (181.6 µM) nanocomposites (inset of fluorescent microscopic structures).