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Ibuprofen causes photocleavage through ROS generation and intercalates with DNA: A combined biophysical and molecular docking approach

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Abstract: Ibuprofen is an important nonsteroidal anti-inflammatory drug endowed with various pharmacological and biological activities. In the present study, the photochemical properties of ibuprofen were evaluated by assaying the generation of various reactive oxygen species (ROS) such as superoxide, singlet oxygen and hydroxyl radical. ROS generated by ibuprofen in presence of white light causes DNA strand scission as observed by plasmid nicking assay. Ibuprofen induced ROS generation is also capable of causing DNA degradation in lymphocytes as observed by photocomet assay. ROS generation properties of ibuprofen was further strengthened by formation of carbonyl groups in BSA and TBARS in linoleic acid as observed by carbonyl assay and lipid peroxidation assay respectively. We have also investigated the mode of interaction of ibuprofen with calf thymus DNA through a series of *in-vitro* experiments. UV-visible spectroscopy established the formation of complex between ibuprofen and Ct DNA. The steady state fluorescence experiments at different temperatures revealed a binding constant of ~10^4 $L.mol^{-1}$ which is an indicative of intercalative binding between ibuprofen and DNA helix. Analysis of the various thermodynamic parameters ΔG, ΔH and ΔS calculated at different temperatures indicated that the hydrogen bonds played major role in the interaction. The intercalative binding mode is further confirmed by Competitive displacement assays, urea denaturation, iodide quenching, viscosity measurement and CD analysis. *In silico* molecular docking revealed the binding of ibuprofen within the GC base pairs of DNA, confirming the intercalative binding mode.

Keywords: reactive oxygen species; non-steroidal anti-inflammatory drugs; fluorescence spectroscopy; intercalation; molecular docking

1. Introduction

Ibuprofen (Fig. 1a), α-methyl-4-(2-methylpropyl) benzene-acetic acid, is one of the arylpropionic acid derivatives of non-steroidal-anti-inflammatory-drugs (NSAIDs) with anti-inflammatory, analgesic and antipyretic activity.[1] A common characteristic of propionic acid derivatives is their ability to generate reactive oxygen species (ROS) in the presence of light. Ibuprofen belongs to this class and was chosen for the phototoxic study because of its capability to generate ROS upon photo-illumination.[2] It has been reported in numerous cases that drug-DNA photoreaction cause undesirable effects. Several classes of drugs such as NSAIDs,[3] phenothiazines,[4] antibacterial fluoroquinolones,[5] other organic chromophore,[6] act as photosensitizers and makes the DNA molecule susceptible to be damaged upon exposure to light.[7] These photoactive compounds give rise to local oxidative stress and cause damage to DNA, proteins and lipids within the cell which induce phototoxic or photogenotoxic effects.[8]

In photoillumination events, the absorption of photon energy by the photoexcited chromophore could be a key trigger for the photosensitization process, followed by the formation of ROS such as superoxide, hydroxyl radical and singlet oxygen. These ROS are recognized as the principal mediators in the drug-induced phototoxic cascades.[9] For the prevention of phototoxic effects screening at the early stage of the drug discovery process and even before the introduction of drugs into clinical therapy is necessary.

In comparison to the drugs that target proteins, the number of known drugs that target DNA is still very limited.[10] Modification in DNA sequence and constellation caused by the interaction of drug molecules is very important to the prevention of disease and improvement of the medical efficacy of drugs.[11] In molecular biology, many synthetic or natural drugs small molecules serve as analogues of interacting entities in protein-nucleic acid recognition, and as sensitive probes for monitoring nucleic acid structure. Consequently, binding of small molecules to DNA interfere with several processes including replication and transcription.

Such restriction can not only prevent cell proliferation, but also have other deleterious effects.[12] Knowing the importance of drug-DNA interaction, detailed investigation of interaction of ibuprofen with DNA was performed, in order to explain the photocleavage activity and mode of interaction which will help us designing better and more effective drugs.

In the present study, the phototoxic potential of ibuprofen was evaluated through analytical and biochemical methods. We have evaluated spectrophotometrically the generation of superoxide, singlet oxygen, and hydroxyl radicals by ibuprofen, upon exposure to light. Plasmid nicking assay, thiobarbituric acid reactive substances assay and photocomet assay were performed to investigate the photogenotoxic potential of ibuprofen. We have also determined carbonyl group formation and peroxidation of linoleic acid to evaluate the role of ROS in ibuprofen induced phototoxic response. Interaction of ibuprofen with calf thymus DNA was also studied to get insight into the binding mode. Various biophysical techniques were employed, which confirmed that ibuprofen binds to DNA by intercalation. Further, molecular docking also confirmed the findings obtained by the biophysical techniques. The combined use of ROS and DNA-binding assays could be fast and reliable promising tool for the detection of the photogenotoxic potential of a large number of drug candidates at the early stage of drug-discovery process.

2. Experimental

2.1. Materials

Ibuprofen sodium salt and calf thymus DNA (Ct DNA) were purchased from Sigma Aldrich, USA. Ethidium bromide (EB) was purchased from Himedia, India. Plasmid pBR322 DNA was purified according to the method described earlier.[13] All reaction mixtures contained 10 mM Tris-HCl (pH 7.2) or else mentioned. All other chemicals were of analytical reagent grade and used without further purification.

2.2. Ct DNA solution preparation

The stock solution of Ct DNA was prepared by dissolving DNA in 10 mM Tris-HCl buffer (pH 7.2) at room temperature with occasional stirring to ensure the formation of a homogeneous solution. Purity of the DNA solution was determined by taking the absorbance ratio of A_{260}/A_{280} which was found to be in the range 1.8-1.9, indicating that the Ct DNA was free from protein and no further purification was needed. The concentrations of Ct DNA solutions per nucleotide was measured as described earlier [14] by using its known extinction coefficient (6600 M^{-1} cm^{-1}) at 260 nm.

2.3. Irradiation procedure

Samples were irradiated with a fluorescent lamp from a distance of 10 cm. At this point spectral irradiation of the source was 38.6 W/m^2 as measured by a power meter (model: Lasermate coherent, USA). There was no measurable change in temperature of the solution at the end of irradiation after two hours at room temperature.

2.4. Determination of reactive oxygen species

2.4.1. Superoxide generation assay (NBT assay)

Generation of superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described earlier.[15] The assay mixture contained 50 mM sodium phosphate buffer (NaPB, pH 7.8), 0.3 mM NBT, 0.1 mM EDTA, and 0.06% Triton-X-100, followed by the addition of ibuprofen ranging from (0-300 μM). Total volume was made to 3 ml by adding NaPB. After mixing, absorbance was recorded at 560 nm using a suitable blank.

2.4.2. Singlet oxygen assay (RNO bleaching method)

Generation of singlet oxygen was studied as described earlier.[16] The sample mixture contained 50 μM p-nitrosodimethylaniline (RNO), 50 μM imidazole and (0-300 μM)

ibuprofen in 20 mM NaPB (pH 7.8). Samples were irradiated with white light and the absorbance was recorded at 440 nm.

2.4.3. Hydroxyl radical determination assay

Hydroxyl radical formation was assayed by the method described earlier.[17] Ct DNA (300 µg) was used as a substrate in a total volume of 3 ml. Increasing concentration of ibuprofen (0-300 µM) was added to each tube. Incubation was done for 2 h at 37^{o}C. Malondialdehyde (MDA) generated from deoxyribose radicals reacts with thiobarbituric acid (TBA) to give coloured complex which was measured by recording the absorbance at 532 nm.

2.5. ROS induced damage studies

2.5.1. Plasmid nicking assay.

In order to examine ROS induced generation of nicks in double stranded DNA in presence of ibuprofen, the plasmid nicking assay was performed. Reaction mixture (25 µl) contained 0.5 µg plasmid DNA, 10 mM Tris-HCl (pH 7.2) and varying concentrations of ibuprofen. Incubation was done for 2 h at 37^{o}C in presence of white light. After incubation, 5 µl of 6X solution containing (40 mM EDTA, 0.05% bromophenol blue tracking dye and 50% (v/v) glycerol) was added and then subjected to electrophoresis on a 1% (w/v) agarose gel. The gel was stained with ethidium bromide and photographed on a UV transilluminator.

2.5.2. Determination of TBARS generation in lymphocytes

Thiobarbituric acid reactive substance (TBARS) was determined according to the method described earlier.[18] Lymphocytes (~1×10^{5} cells/ml) were incubated with ibuprofen (0–300 µM) at 37°C for 2 h in presence and absence of white light and then centrifuged at 5000 rpm. The cell pellet was washed twice with phosphate buffer saline (Ca^{2+} and Mg^{2+} free) and resuspended in 0.1 N NaOH (1.0 ml). In order to stop the reaction, the cell suspension was further treated with 10% trichloroacetic acid (TCA). Finally, 0.6 M TBA was added and

samples were incubated in a boiling water bath for 10 min followed by measuring the absorbance at 532 nm.

2.5.3. Photo comet assay

In an attempt to examine the phototoxic effect of ibuprofen on cellular DNA, comet assay was performed as described earlier.[19,20] Fresh blood samples (3 ml) were collected from healthy volunteers by vein puncture and stored in presence of heparin to avoid clotting. Lymphocytes were isolated from the diluted blood using Histopaque 1077 (HiMedia) and suspended in RPMI 1640. Trypan Blue Exclusion test [21,22] was performed before start and after the end of experiment to check the viability of lymphocytes. Lymphocytes (~1×10^5 cells) were exposed to different concentrations of ibuprofen in a total reaction volume of 500 µl that also included Ca^{2+} and Mg^{2+} free PBS and RPMI 1640. Incubation was done for 2 h at 37^{o}C in absence and presence of light and then mixture was centrifuged at 5000 rpm to collect the lymphocyte. The cell pellet was further suspended in 100 µl Ca^{2+} and Mg^{2+} free PBS and further processed for the comet assay. Comet tail length of 50 nuclei were analyzed and tail length was calculated using image analysis software (Komet 5.5; Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope (Olympus Optical Co, Tokyo, Japan) and a COHU 4910 integrated CC camera equipped with 510-560 nm excitation and 590 nm barrier filters (COHU, San Diego, CA, USA). Migration of DNA from the nucleus i.e. tail length was measured as the main parameter to assess lymphocyte DNA damage.

2.5.4. Carbonyl group formation assay

Oxidative modification of BSA by ibuprofen generated ROS was studied by calculating total carbonyl group formation. After incubating BSA (1 mg/ml) with the desired concentration of ibuprofen (0-300 µM) for 2 h in absence and presence of white light, the amount of carbonyl groups formed was determined by the method described earlier. [23] Briefly, BSA was

incubated for 2 h with varying concentration of ibuprofen and then protein was precipitated using 20% TCA followed by centrifugation. Pellet of the sample was dissolved in 0.5 ml 2,4-Dinitrophenylhydrazine and placed on shaking rocker for 1 h and again 20% TCA solution was added to the tube followed by centrifugation at 12,000 rpm for 15 min. Supernatant was discarded and the protein pellets were washed thrice using ethanol:ethyl acetate (1:1, v/v) and finally dissolved in 6 M guanidine HCl (pH 2.3). Absorbance was recorded at 360 nm and calculation was done using molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

2.5.5. Photosensitized peroxidation of linoleic acid

Lipid peroxidation was assessed using a TBA assay as described earlier. [24] A typical assay mixture contained linoleic acid (1 mM) suspended in 20 mM NaPB (pH 7.8) containing 0.05% Tween 20. Reaction mixture was irradiated with light in the presence of desired concentration of ibuprofen (0-300 μM). To the irradiated sample (500 μL), 1 ml of 0.67% TBA (dissolved in 20 mM NaPB, pH 7.8) and 10 μL of 1.0% butylated hydroxytoluene solution in glacial acetic acid were added, followed by heating at 95°C for 30 min. After cooling at room temperature, the mixture was extracted with 1.0 mL of 1-butanol. The absorbance of the extract was measured at 532 nm for the determination of TBARS, which was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.6. Drug-DNA interaction studies

2.6.1. UV spectroscopic method

The measurements were performed on a Beckman DU 40 spectrophotometer (USA) using a 1×1 cm quartz cuvettes. The UV-spectra of ibuprofen complexed with DNA were measured in the wavelength range from 250-300 nm. Experiment was carried out by keeping the fixed amount of ibuprofen 50 μM in a total volume of 3 ml and was subsequently titrated with increasing concentration (0-10 μM) of Ct DNA. DNA solutions of same concentrations

without ibuprofen were used as the blank so that the UV-spectra specific to ibuprofen-DNA complex could be analyzed.

2.6.2. Fluorescence Measurements

All fluorescence spectra were recorded on a Shimadzu spectroflurometer-5000 (Japan) equipped with xenon flash lamp using 1.0 cm quartz cells. The emission spectra of ibuprofen were obtained in the range 270 to 330 nm upon excitation at 264 nm with the excitation and emission slit width of 5 nm each. Ibuprofen concentration was set at 50 μM in a reaction mixture of 3 ml and the DNA concentration was varied from 0-10 μM.

2.6.3. Displacement assays

EB displacement assay was performed using a solution containing (2 μM) EB and (50 μM) DNA, which was titrated with increasing concentration of ibuprofen. EB-DNA complex was excited at 475 nm and emission spectra were recorded from 550-620 nm. In another similar experiment DNA-Hoechst 33258 complex was excited at 343 nm and was subsequently titrated with increasing concentration of ibuprofen. Emission spectra were recorded in the range of 350-600 nm.

2.6.4. Potassium iodide (KI) quenching method

KI induced quenching of fluorescence intensity was performed in presence and absence of DNA. Ibuprofen (50 μM) was taken and titrated with varying concentration of KI (0-72 mM). In another set, ibuprofen (50 μM), Ct DNA (13.5 μM) were taken and KI was added sequentially (0-72 mM). Volume of the reaction mixture was made to 3 ml by adding 10 mM Tris-HCl (pH 7.2). Excitation was done at 264 nm and emission spectra were recorded between 270-500 nm. Fluorescence quenching efficiency was evaluated using the Stern-Volmer equation.

2.6.5. Effect of urea

Urea induced denaturation study has been exploited to determine the interaction between Ct DNA and ibuprofen. This assay was carried out by using fixed concentration of ibuprofen (50 μM), and Ct DNA (13.5 μM) in a total volume of 3 ml, while varying the concentration of urea (0-2.66 M). Excitation was done at 264 nm and emission spectra were recorded between 280-310 nm.

2.6.6. CD studies

CD measurements of DNA in the absence and presence of ibuprofen was performed in the range of 225-300 nm on an applied Photophysics spectrophotometer equipped with a Peltier temperature controller (U.K., model CIRASCAN). All the CD spectra were collected with a scan speed of 200 nm/min with a spectral band width of 10 nm. Each spectrum was the average of four scans. The molar ratio of DNA concentration to drug concentration was 1:0, 1:1 and 1:2.

2.6.7. DNA melting studies

UV-visible spectroscopy is a simple method for determining the melting temperature of DNA double helix. DNA melting experiments were carried out by monitoring the absorption of Ct DNA sample at various temperatures in absence and presence of ibuprofen. The samples contained either 50 μM Ct DNA alone or in the presence of 50 μM of ibuprofen. The volume of the sample was made upto 3 ml using Tris-HCl buffer (pH 7.2). The temperature of the sample was continuously monitored with a thermocouple attached to the sample holder. The absorbance was taken at 260 nm and then plotted as a function of temperature ranging from 25 to 100°C. The *T*m of DNA was determined as the transition midpoint.

2.6.8. Viscosity Measurement

Viscosity experiments were carried out with an Ubbelohde viscometer (Canon, Model-9721-K56, Coleparmer, USA) suspended vertically in a thermostat water bath at room temperature. Viscosity measurements were carried out made by keeping the concentration of DNA constant (100 μM) and varying the concentration of the ibuprofen. The flow time was measured repeatedly by using a digital watch with an accuracy of (±0.20 s). The viscosity of the DNA solution was measured in the presence of increasing amounts of ibuprofen. The data were reported as $(\eta/\eta_o)^{1/3}$ versus the binding ratio, where η is the viscosity of DNA in the presence of ibuprofen and η_o is the viscosity of DNA solution alone as described earlier.[25]

2.6.9. Molecular docking

The molecular docking studies were performed using Hex 8.0.0 [26] to understand the ibuprofen-DNA interactions. HEX is an interactive molecular graphics program for calculating and displaying feasible docking modes of DNA. The 3D structure of the B-DNA dodecamer $[d(CGCGAATTCGCG)]_2$ was downloaded (PDB ID 1BNA) from the Protein Data Bank (http://www.rcsb.org./pdb). The structure-data file of ibuprofen was obtained from https://pubchem.ncbi.nlm.nih.gov/ and converted into PDB format using Avagadro's 1.01. [27] Hex 8.0.0 performs docking using spherical polar Fourier correlations, and requires the ligand and the receptor to be input in PDB format. The parameters used for docking include: correlation type, shape only; FFT mode, 3D; grid dimension, 0.6; receptor range, 180; ligand range, 180; twist range, 360; distance range, 40. Visualization of the docked pose was performed using PyMol (DeLano Scientific, San Carlos, CA, USA).

2.7. Statistical analysis

Results are expressed as mean ±SD of at least three independent observations. A Student's t-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. p values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. ROS generation by ibuprofen

There is an increase in the usage of topical gels containing NSAIDs (ibuprofen, ketoprofen, or diclofenac) against aching joints or muscle pains.[7] Concentrations of NSAIDs in these topical gels were found to be around 5% to 10% (w/w), [28] which is equivalent to 219 mM to 438 mM of ibuprofen. Considering the concentrations of ibuprofen used in these topical gels, we have used 300 to 500 times less concentration of ibuprofen in our study to asess the phototoxicity.

The generation of singlet oxygen, superoxide and hydroxyl radicals were studied from photoirradiated ibuprofen and were observed spectrophotometrically. These radical species act as major harmful mediators in the downstream of drug-induced phototoxic cascades [29] and are responsible for the oxidative damage against various biomolecules including DNA, proteins and lipids. To determine the ability of ibuprofen to generate ROS, several assays were performed.

In NBT assay, as apparent from the (Fig. 1b), photoirradiation of ibuprofen led to the generation of superoxide anion in a concentration-dependent manner.[15,16] Upon photoillumination ibuprofen generates superoxide anion, that reduces NBT via a one-electron transfer reaction, producing partially reduced monoformazan (NBT^{+}) as a stable intermediate, which was recorded at 560 nm using a suitable blank.

Detection of singlet oxygen ($^1O_2$) was done by RNO bleaching method. The degree of photobleaching of the yellow RNO dye is directly proportional to the production of $^1O_2$ generated in the photodynamic reaction, which can be detected spectrophotometrically as a decrease in the absorbance of the dye at 440 nm. [15,16] Since the singlet oxygen does not react chemically with RNO, this bleaching is caused by the capturing of $^1O_2$ by the imidazole ring, which results in the formation of a transannular peroxide intermediate, causing the bleaching

of RNO. Generation of singlet oxygen from irradiated ibuprofen seems to be concentration-dependent (Fig. 1c).

Hydroxyl radical generation by ibuprofen was analysed in absence and presence of white light. As seen in (Fig. 1d), increasing the concentration of ibuprofen in presence of white light leads to the concentration dependent formation of hydroxyl radicals. However, in the absence of light the generation of hydroxyl radicals was negligible.

The above results clearly demonstrated the ROS generation capability of ibuprofen in presence of white light.

3.2. Phototoxic potential of ibuprofen

3.2.1. Plasmid nicking assay

Plasmid nicking assay is a very sensitive method to detect any DNA damage caused due to ROS generation. In fact, only one single-strand break is enough to cause the structural transition of the supercoiled form of plasmid into the relaxed form while double strand breaks lead to the formation of linear form. [26] All three forms are easily separated on agarose gel. Ibuprofen did not promote DNA strand breaks in absence of white light irradiation. However, treatment of plasmid DNA with ibuprofen in the presence of white light showed relaxation in supercoiled plasmid DNA. The increase in the intensity of the open circular form and appearance of a DNA band corresponding to linear DNA depicts the plasmid DNA strand breaks activity of ibuprofen (Fig. 2).

3.2.2. Lymphocytes DNA damage

DNA damage caused by oxygen radicals give rise to TBA-reactive substances abbreviated as TBARS. [30,31] We have therefore, determined the formation of TBARS as a measure of oxidative stress in lymphocytes with increasing concentration of ibuprofen. As seen in Fig. 3a, concentration dependent TBARS formation was observed in the absence and presence of

white light. However formation of TBARS was more prominent in presence of white light (Fig. 3a).

3.2.3. Photo comet assay

To further elucidate the role of ROS in DNA damage, comet assay was performed using human lymphocytes. Comet assay is a sensitive technique to detect DNA damage caused by photosensitizing chemicals. [32,33] Fig. 3 shows comets of lymphocyte cells treated with increasing concentration of ibuprofen (0-300 μM), with and without irradiation of white light. Without irradiation the degree of DNA damage was less (Fig. 3b) as compared to damage induced by ibuprofen in presence of white light. At 50 μM (Fig. 3c) and 300 μM (Fig. 3d) concentrations in absence of white light ibuprofen did not damage the lymphocyte DNA to significant extent. However, degree of DNA damage in presence of white light at 50 μM (Fig. 3e) and 100 μM (Fig. 3f) was enhanced. Photoirradiation of cells treated with ibuprofen led to the induction of hedgehog comets (Fig. 3f) with a small head and a strong tail (i.e. high degree of DNA migration). Tail length obtained in presence of increasing concentration of ibuprofen in absence and presence of light is reported in Fig. 3g. The damage caused by ibuprofen was more prominent in presence of light. [52] From the results obtained by comet assay, it is deduced that photoactivation of ibuprofen generates ROS which is capable of causing extensive DNA damage.

3.2.4. Carbonyl assay and lipid peroxidation

In order to further study the ROS induced damage to other biomacromolecules by ibuprofen, protein damage by carbonyl assay and lipid peroxidation assay was performed.

Proteins are target for oxidative modification by reactive oxygen species. Carbonylation of protein is one of the irreversible and important modifications that increases during oxidative stress. [34] As evident in Fig. 4a, with increasing concentration of ibuprofen in presence of white light there was an increase in the formation of carbonyl groups. This

clearly indicated ROS generation property of ibuprofen in presence of light since oxidative damage to protein was found to be negligible in absence of light (Fig. 4a).

Lipid peroxidation is considered to be one of the major mechanisms in phototoxic responses induced by several NSAIDs.[35] Photoirradiation of ibuprofen in the presence of linoleic acid generates free radicals, which led to the formation of secondary products called as TBARS. [36] TBARS assay measures MDA present in the sample, as well as MDA generated from fatty acid hydroperoxides. [37] As observed (Fig. 4b) in the absence of ibuprofen, irradiation of linoleic acid (1 mM), yielded a small amount of peroxidation products, but irradiation in the presence of ibuprofen (0-300 μM) results in greater amount of such products, suggesting that generation of lipoperoxidants in the presence of ibuprofen occurs in a concentration dependent manner. [15,16] In contrast, in the absence of white light increasing the concentration of ibuprofen did not lead to enhanced lipid peroxidation.

On the basis of our experiments, we proposed a tiered strategy for phototoxicity/photosensitivity testing. First, ROS generation assays could be used for initial screening, which allows the detection of the ROS generation capabilities of drugs. Plasmid nicking assay, TBARS assay, comet assay, carbonyl assay and lipid peroxidation could be used for the second stage screening to assess the phototoxic potential of the compounds. Based on the results obtained, we conclude that upon photoillumination ibuprofen generates ROS causing damage to various bio-macromolecules. This suggested that the current study may be useful to assess the photosensitive and phototoxic potential of testing compounds with ease and high productivity.

3.3. Interaction of ibuprofen with DNA

3.3.1. UV-visible spectroscopy

UV-visible spectroscopy is a simple and effective method to detect drug-DNA interaction where changes in absorbance intensity and any shift in the wavelength are recorded. In order

to investigate the interaction between ibuprofen and Ct DNA, the UV-visible absorption spectra of ibuprofen in the presence of different concentrations of Ct DNA were measured (Fig. 5). In the absorption spectra of ibuprofen, band centered at ~264 nm and ~274 nm exhibits large hyperchromism (increase in absorbance) on continuous addition of Ct DNA, suggesting the formation of adduct between Ct DNA and ibuprofen. [26] It is evident from (Fig. 5) that there is no clear isobestic point, suggesting that 1:1 binding stoichiometry is not maintained during the process or there may be more than one mode of binding. Hence, from UV spectra, we can conclude that there is a formation of drug-DNA complex. However, it does not give any clear idea about the mode of interaction, so further experiments were done to get into the details of interaction mode.

3.3.2. Steady state florescence

Fluorescence experiments are often employed to study the mode of drug-DNA interactions. Thus, for the determination of DNA-binding activity of ibuprofen, the steady state fluorescence technique was applied. Fig. 6a shows the emission spectra of ibuprofen and its fluorescence titration with Ct DNA. The drug has an emission spectrum with maxima centered at ~292 nm when excited at 264 nm (Fig. 6a). In the presence of increasing DNA concentration, a progressive quenching of the fluorescence intensity was observed without any shift in the wavelength, indicating the strong interaction between ibuprofen and Ct DNA. [26,43] It is known that the intercalation of small molecules into Ct DNA base pairs leads to a restriction in their rotational motion and favour radiationless deactivation of the excited states (loss of excitation energy without photon emission). However, if these drugs are merely bound to the phosphate backbone or the grooves, the deactivation by fluorescence emission is favoured leading to a significant increase in the fluorescence emission. [38] In case of ibuprofen-DNA interaction we have observed the significant quenching with increasing concentration of DNA, suggesting that the binding mode should be intercalative.

The fluorescence quenching data were analyzed to obtain the fluorescence quenching (K_{sv}) constant by using the Stern-Volmer equation. The Stern-Volmer quenching plot from the fluorescence titration results is shown in (Fig. 6b).

$$(F_0/F)=1+K_{sv}[Q] \quad (1)$$

where F_0 and F represent the fluorescence intensities in the absence and presence of the DNA (Q) respectively and K_{sv} is the Stern-Volmer quenching constant, which is a measure of the efficiency of quenching by DNA. The slopes of the (F_0/F) vs [DNA] plots yield the values of K_{sv} and it was calculated to be of the order of 10^4 L.mol^{-1} (Table 1), which was found to be consistent with intercalative binding mode. [14,39] Thus, ibuprofen is suggested to interact with DNA by intercalative mode.

The Stern-Volmer plot is linear, indicating that out of two quenching processes (static and dynamic) only one type of quenching process occurs. [40] The process of quenching was further confirmed from the values of the bimolecular quenching rate constants (K_q) which are evaluated using the equation:

$$K_q = K_{sv}/\tau_0 \quad (2)$$

where τ_0 is the average lifetime of molecule without quencher. Since fluorescence lifetimes are typically near 10^{-8} s, Kq was calculated from the above equation and was found to be in the range of 10^{12} L. mol^{-1} s^{-1} (Table 1). Apparently, the value of K_q was greater than the value of the maximum scatter collision quenching constant (2.0×10^{10} L. mol^{-1} s^{-1}). [41] Dynamic quenching can be distinguished from static quenching by calculating K_{sv} at different temperatures. In dynamic quenching, higher temperatures can lead to faster diffusion and increased collisional quenching, so K_{sv} increases with increasing temperature. However, in static quenching higher temperatures will typically cause the dissociation of weak-bound

complexes, so K_{sv} decreases with increasing temperature. [42] The trend in the present study demonstrated the decrease in K_{sv} with increasing temperature (Table 1) indicated that the probable quenching mechanism of ibuprofen fluorescence by DNA is static type.

3.3.3. Binding equilibrium

For static quenching, the relationship between fluorescence intensity and concentration of a quencher can be described by the following equation. [43]

$$\log[(F_0\text{-}F)/F] = \log K + n \log[Q] \quad (3)$$

where K and n designate the binding constant and the number of binding sites, respectively. From the plot of log $(F_0\text{-}F)/F$ vs. log [Q], K and n values can be obtained from the intercept and slope (Figure 6c). The decreasing trend of K, and n with increasing temperature from 293 K to 313 K (Table 1) was in accordance with K_{sv}'s values obtained above. This is possibly due to reduced stability of ibuprofen-DNA complex at higher temperature.

3.3.4. Determination of thermodynamic parameters

The thermodynamic parameters ΔH and ΔS were evaluated from the equation shown below.[44]

$$\log K = -\Delta H^0 / 2.303RT + \Delta S^0/2.303R \quad (4)$$

where R is the universal gas constant and T is the absolute temperature. The binding constant (K) values were determined at four different temperatures as described above. A van't Hoff plot of lnK against 1/T was plotted (Figure 6d) to calculate the slope and intercept of the plot, ΔH and ΔS respectively. The values of the thermodynamic parameters are listed in Table 2. The free energy change (ΔG) is calculated from the following equation.

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

The negative sign values of (ΔG) means that the binding process is spontaneous. The values in change in negative enthalpy (ΔH) and entropy (ΔS) during the interaction of ibuprofen and

DNA indicated that the hydrogen bonds and van der Waals interactions played a major role in binding process.

3.4. Binding mechanism of ibuprofen and DNA

3.4.1. Displacement assays

An exclusion assay with ethidium bromide (EB) has been implicated to obtain the information about the ability of any small molecule to displace EB from its EB-DNA complex. EB emits intense fluorescence in presence of DNA due to its strong intercalation between the adjacent DNA base pairs. [45] The displacement of EB on addition of a drug is suggestive of an intercalative binding mode. Any small molecule replacing the EB from DNA will result in decreased flouresence intensity. In experiment with ibuprofen, a decrease in the fluorescence intensity of EB-DNA complex was observed on subsequent addition of ibuprofen (Fig. 7a). This shows that ibuprofen is able to displace EB from DNA helix and strongly competes with EB for intercalation sites of DNA helix. [45] To further confirm the binding mode we used Hoechst 33258, which binds to the minor groove of double stranded B-DNA.[46] Groove binding molecules are able to displace Hoechst 33258 from the minor groove of the DNA helix, resulting in decreased fluorescence intensity of DNA-Hoechst complex. On addition, of ibuprofen, there was no change in the fluorescence intensity of the Hoechst-DNA complex (Fig. 7b). [52] Thus, we conclude that the binding mode of ibuprofen with Ct DNA is intercalative binding. The intensity plot i.e. relative extent of fluorescence quenching in the absence and presence of ibuprofen (F/F_0) has been plotted as a function of ibuprofen concentration for EB and Hoescht-DNA system (Fig. 7c).

3.4.2. KI quenching studies

To further reveal the binding mechanism of ibuprofen to DNA, iodide quenching experiments were employed. In this study, negatively charged potassium iodide (KI) was taken, since it can effectively quench the fluorescence intensity of small molecules. Because

of the repulsion between the anionic quencher and negatively charged phosphate backbone of DNA, any small molecule intercalated within the DNA helix would experience protection from anionic quencher, whereas drugs having groove binding properties should be quenched in presence of anionic quenchers, since groove binding exposes the bound molecules to the solvent surrounding the DNA helix much more than does the intercalation. [47,48] Further, K_{sv} was calculated from the equation (1) using Stern-Volmer plots as shown in Fig. 8a. The calculated K_{sv} values for ibuprofen by KI in the absence and presence of DNA was found to be 17.9 and 8.2 $mol \cdot L^{-1}$, respectively. [47] Table 3 summarizes the calculated K_{sv} from Stern-Volmer plots. The low K_{sv} value for ibuprofen in the presence of DNA, suggested that the Ct DNA bound ibuprofen is sequestered away from the solvent suggesting intercalative binding inside the DNA helix.

3.4.3. Effect of Urea

The property of urea to destabilize the double stranded DNA helix is exploited in studying the binding mode of small molecules with DNA.[49] The addition of denaturants results in the release of entrapped drug molecules from DNA strands into buffer solution leading to modification in the fluorescence behaviour of the drug molecules. [50] Gradual addition of urea to the ibuprofen Ct DNA complex resulted in the progressive enhancement in the fluorescence intensity (Fig. 8b) indicating the release of the DNA bound ibuprofen molecule to the buffer solution. This suggests that urea is able to liberate ibuprofen from within the DNA strands and hence provides evidence in support of the intercalative mode of binding. [47] The relative extent of intercalation of ibuprofen was given by the ratio of peak fluorescence intensities in the presence and in the absence of urea (F/F_0) and has been plotted as a function of urea concentration, is depicted in (Fig. 8b).

3.4.4. Circular dichroism studies

CD spectroscopy is a sensitive technique which detects any conformational changes in DNA on addition of a ligand. We measured CD spectrum of Ct DNA in presence of various concentrations of ibuprofen. Non covalent DNA-drug interactions affect the structure of the DNA, and hence alter their intrinsic CD spectral behavior. [51] The observed CD spectrum of Ct DNA consists of two major peaks at 245 nm (negative) and 275 nm (positive) that are assigned to right handed helicity of B-DNA and base pair stacking respectively. [26,52] There is negligible perturbation of the base stacking and helicity bands in case of interaction of non-intercalative drugs with DNA, whereas an intercalator changes the intensities of both the bands. [53,54] As evident from (Fig. 9a), with increasing concentration of ibuprofen, the intensity of both the bands were changed. Intercalation of ibuprofen disrupts the stacked nitrogenous bases within the DNA double helix, which leads to a decrease in the peak elipticity at ~275 nm. [55] Little change in the intensity of the CD peak at 245 nm occurs due to the alteration in the hydration layer of the DNA helix. These changes may thus reflect that in order to accommodate the intercalated ibuprofen within a particular base pair the relative orientation of DNA bases is adjusted. Thus, from the results of UV-visible absorption spectroscopy, fluorescence spectroscopy, viscosity measurements and CD spectroscopic studies, we conclude that the ibuprofen binds to DNA primarily in an intercalative fashion.

3.4.5. DNA melting studies

Another reliable method to investigate the interaction of small molecules with DNA is to study the thermal melting profile of DNA-drug complex. Interactions of small molecules with the DNA double helix are known to influence the melting temperature (Tm), the temperature at which the double helix denatures into single stranded DNA. [56] Intercalation of small molecules can stabilize the double helix structure causing increase in the Tm by about 5-8 °C, but groove binding and electrostatic binding causes less or no obvious increase in Tm. [14] The value of Tm for Ct DNA was determined by monitoring the absorbance of the systems at 260

nm as a function of temperature ranging from 25 to 100°C. For each monitored transition, The Tm of the assay solution, was determined as the transition midpoint of the melting curve. The melting curves of Ct DNA in absence and presence of ibuprofen is shown in Fig. 9b In presence of increasing concentration of ibuprofen, the Tm of Ct DNA remarkably increased from the initial 66.8 ± 1°C to 73 ± 1°C, revealing that binding mode of ibuprofen to Ct DNA is intercalation. [56]

3.4.6. Viscosity measurement

To further investigate the interaction between the ibuprofen and DNA, viscosity measurements were carried out. Viscosity measurement is a very sensitive and reliable method which records any change in DNA length upon addition of a compound. [57] In general, a classical intercalation causes an increase in the viscosity of DNA because in order to accommodate the binding ligand, separation of the DNA base pairs would take place which leads to an increase in the length of DNA helix. However, groove binding and electrostatic binding could bend and kink the DNA helix, thereby reducing its effective length and consequently its viscosity. [26,58] As shown in Fig. 9c a gradual increase in the relative viscosity upon addition of ibuprofen suggested that the nature of binding is intercalative. [57]

3.4.7. Molecular docking

Molecular docking technique is an attractive tool to decipher the drug-DNA interactions in rational drug design and also to get insight of the mechanistic study, by placing a molecule into the binding site of the target specific region of the DNA.[59,60] The structure of DNA and ibuprofen was obtained from Protein Data Bank (PDB) (http://www.rcsb.org/pdb). In order to predict the best fit orientation of ligand within the DNA helix, ligands are made flexible to attain different conformations, followed by the analysis of best energy docked poses. Several runs of search were carried out to determine the best ligand-receptor orientation. In our experiment, rigid molecular docking studies were performed which keeps the DNA structure

rigid while allowing the ligand to change its conformation to attain the best ligand receptor orientation. This was performed to predict the binding mode of ibuprofen with a DNA duplex of sequence $[d(CGCAAATTTCGC)]_2$ dodecamer (PDB ID:1BNA) that provide an energetically favorable docked structures. As evident (Fig. 10), ibuprofen binds to DNA by intercalating within the nucleotide base pairs of DNA. [26,59] The resulting relative binding energy of docked DNA-drug complex was found to be ~–5.682 kcal/mol which is of the same order of magnitude as was obtained from steady state fluorescence studies. Docking results have clearly shown the formation of a hydrogen between the oxygen bearing group (O 1) of ibuprofen and tenth guanine of one strand of DNA (A chain of DNA) (Figs. 10a, 10b), with the nitrogen (N2) atom serving as a hydrogen bond receptor (Fig. 10c). Distance between the hydrogen bond is 2.8 Å (Fig. 10d). Irrespective of the electrostatic repulsion between ibuprofen and DNA bearing same negative charges, large negative value of the binding energy indicated a higher binding potential of the ibuprofen with DNA. It has been well reported that groove binders, preferably bind to AT rich sequences, whereas intercalators preferably bind to GC rich sequences. [61] The analysis of the docking results further revealed the binding of the drug at the GC rich region, revealing the intercalative mode of binding of ibuprofen (Fig. 10e). [26] Therefore, we conclude that there is a mutual coherence between spectroscopic techniques and molecular docking in ibuprofen-DNA interaction.

4. Conclusion

This study clearly demonstrated the generation of ROS by ibuprofen in presence of light. The photochemical reaction is associated with their phototoxic effects on biomolecules. Based on the findings attained in this study, the ROS assay approach could be of use as a first screening to classify drugs in the early stage of pharmaceutical development. Our work has also focused on the mode of interaction of ibuprofen with DNA. The intercalative binding of ibuprofen with DNA was deduced by various methods including UV-visible absorption

spectra, fluorescence spectra, displacement assays, KI induced quenching, urea denaturation assay, CD studies and viscosity measurements. The molecular docking results further revealed the intercalative mode of interaction between ibuprofen and DNA. The knowledge gained from this study could be used for the development of potential probes for DNA structure as well as further understanding the pharmacological effects of other compounds.

Notes

The authors declare that there is no conflict of interest in this work.

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Table 1 Various binding constants at different temperatures for ibuprofen-DNA interaction

T (K)	K_{sv} ($\times 10^4$) ($L.mol^{-1}$)	K_q ($\times 10^{12}$) ($L.mol^{-1}s^{-1}$)	K ($\times 10^4$) (mol^{-1})	*n*	$R^{2\,(a)}$
293	9.9	9.9	5.21	1.044	0.9995
301	6.5	6.5	4.92	1.008	0.9998
313	3.3	3.3	4.43	0.939	0.9999

[(a)] R^2 is the correlation coefficient.

Table 2 Various thermodynamics parameters obtained for the binding of ibuprofen with Ct DNA.

T (K)	ΔH (kcal/mol)	ΔS (cal/mol per K)	ΔG (kcal/mol)
293	-	-	-7.21
301	-16.30	-31.68	-6.96
313	-	-	-6.59

Table 3 K_{sv} values of ibuprofen by KI in absence and presence of Ct DNA.

Complex	K_{sv} ($mol \cdot L^{-1}$)	$R^{2\,(a)}$	S.D. [(b)]	Relative reduction in K_{sv} (%)
Ibuprofen+KI	17.9±0.28	0.99852	0.7263	
Ibuprofen+KI+DNA	8.2±0.23	0.99941	0.7841	54

[(a)] R^2 is the correlation coefficient. [(b)] S.D. is standard deviation.

Results shown are the means ± SD of three experiments.

Figure legends

Fig. 1 (a) Chemical structure of Ibuprofen sodium. (b-d) Generation of reactive oxygen species from ibuprofen exposed to white light. (b) Superoxide anion (c) Singlet oxygen (d) Hydroxyl radical. Data represent mean ± S.D. of three experiments.

Fig. 2 Agarose gel electrophoresis pattern of ethidium bromide stained pBR322 DNA after treatment with ibuprofen in presence of white light. Lane 'A' depicts the 'Control' which contain only plasmid DNA. The concentrations of ibuprofen in lanes 'B-F' was 200, 300, 400, 500, 600 μM respectively. Arrows indicating OC, L and SC on the right represent the relaxed, linear and supercoiled forms of plasmid DNA.

Fig. 3 (a) Effect of increasing concentration of ibuprofen on lymphocyte DNA breakage in presence and absence of white light. Assessment of DNA damage was done by measuring the amounts of TBARS using thiobarbituric acid assay. Data represent mean ± S.D. of three determinations. (b-g) Comet assay after treatment with ibuprofen in presence and absence of light. The incubation period was 2 h at 37°C. (b) Control lymphocyte. (c, d) Lymphocyte nuclei showing comets after treatment with ibuprofen (50 and 300 μM respectively) in absence of light. (e, f) Lymphocyte nuclei showing comets with ~~photoilluminated~~ ibuprofen (50 and 300 μM respectively) in presence of light. (g) Comparison of lymphocyte DNA breakage with or without photoillumination while increasing the concentration of ibuprofen (0-300 μM). Data represent mean ± SD of three experiments. *p value <0.05 as compared to control.

Fig. 4 (a) Dose-dependent formation of carbonyl groups. BSA solution was incubated with increasing concentration of ibuprofen for 2 h at 37^0C. In dark carbonyl groups formation was negligible. (b) Linoleic acid (1 mM) and ibuprofen were dissolved in 50 mM NaPB (pH 7.8) and then exposed to white light for 2 h. Lipid peroxidation was measured using a TBA assay. Quantification of the TBARS was done by measuring the absorbance at 532 nm, which was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Values reported are ±SD of three experiments. *p value <0.05 as compared to control.

Fig. 5 UV-Vis absorption spectra of ibuprofen (50 μM) in presence of various concentrations of Ct DNA (0-10 μM) in Tris-HCl buffer (pH 7.2). Spectra were recorded in the range of 250-300 nm. The arrow shows the changes upon increasing amounts of Ct DNA.

Fig. 6 (a) Fluorescence emission spectra of ibuprofen (50 μM) in the presence of various concentrations of Ct DNA. Excitation and emission wavelengths were 264 nm and 270-330 nm respectively. Arrow shows the decrease in intensity upon increasing the concentration of Ct DNA. (b) Fluorescence quenching Stern-Volmer plot of ibuprofen with increasing concentration of DNA. The plot represents the binding of ibuprofen at 293K, 301K and 313K. (c) Binding isotherm of log $[(F_o-F)/F]$ versus log [DNA]. The plot represents the temperature-dependent binding of ibuprofen with DNA. (d) A van't Hoff plot of lnK against 1/T for ibuprofen-DNA system. Data represent mean ± SD of three experiments.

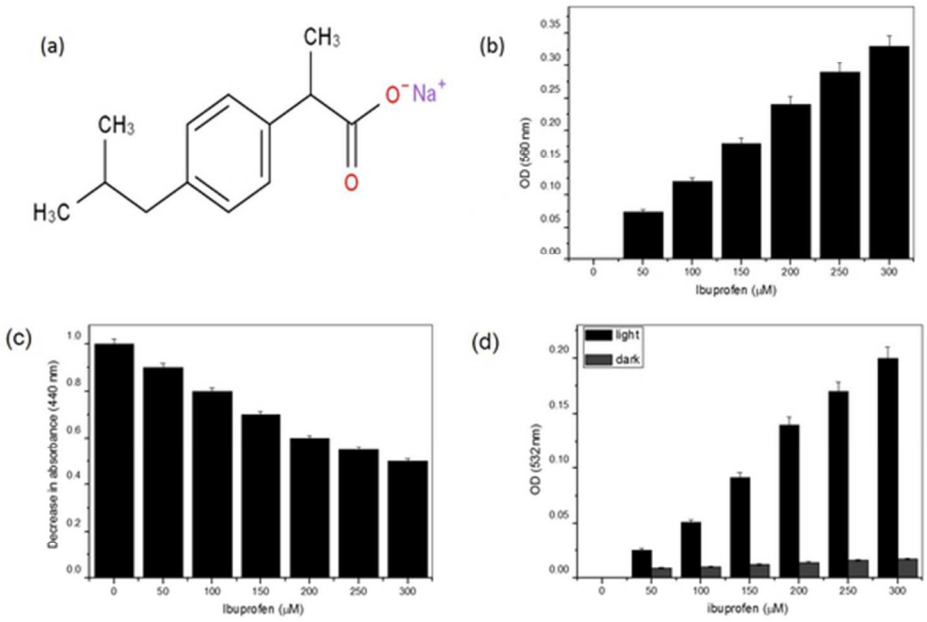
Fig. 7 (a) Ct DNA (50 μM) was dissolved in 10 mM Tris HCl (pH 7.2) and EB was added to a final concentration of 2 μM with excitation and emission wavelength of 475 nm and 550-620 nm respectively. Fluorescence quenching studies were performed with varying concentration of ibuprofen (0-190 μM). (b) Fluorescence titration of Ct DNA and Hoechst (groove binder) complex with ibuprofen. Ct DNA-Hoechst complex was excited at 343 nm and emission spectra were recorded from 350-600 nm. (c) Intensity plot observed at 590 nm for EB and 460 nm for Hoechst vs concentration of ibuprofen.

Fig. 8 (a) Stern-Volmer plot for fluorescence quenching of ibuprofen (50 μM) by KI in absence and presence of Ct DNA (13.5 μM) in 10 mM Tris-HCl buffer (pH 7.2). Concentration of KI was varied from 0 to 72 mM. Data represent mean ± SD of three experiments. *p value <0.05 as compared to control. (b) Fluorescence spectra of ibuprofen with urea. Ct DNA-ibuprofen was excited at 264 nm and emission spectra were recorded from 280-310 nm. Fluorescence intensity increases with subsequent addition of urea. Inset shows the variation of fluorescence intensity of Ct DNA-bound ibuprofen, as a function of urea concentration. Data represent mean ± SD of three experiments.

Fig. 9 (a) CD spectra of Ct DNA (30 μM) in 10 mM Tris-HCl (pH 7.2) with varying concentration of ibuprofen. Each spectrum was obtained at 25°C with a 10 mm path length cell. (b) Melting curves of Ct DNA (50 μM) in absence and presence of ibuprofen (50 μM). (c) Effect of increasing concentration of ibuprofen on the viscosity of Ct DNA. Concentration of DNA was kept constant while varying the ibuprofen concentration. Data represent mean ± SD of three experiments. *p value <0.05 as compared to control.

Fig. 10 Different poses of molecular docked structure of ibuprofen complexed with DNA showing (a and b) the formation of a hydrogen bond between the oxygen bearing group (O 1)

of ibuprofen and tenth guanine of A chain (c) nitrogen atom serving as a hydrogen bond receptor (d) the distance of hydrogen bond between ibuprofen and A chain (O----H: 2.8 Å) and (e) binding of ibuprofen to GC region of dodecamer duplex of sequence [$(CGCGAATTCGCG)_2$ (PDB ID: 1BNA)]. The relative binding energy of the complex system was found to be -5.682 kcal/mol.



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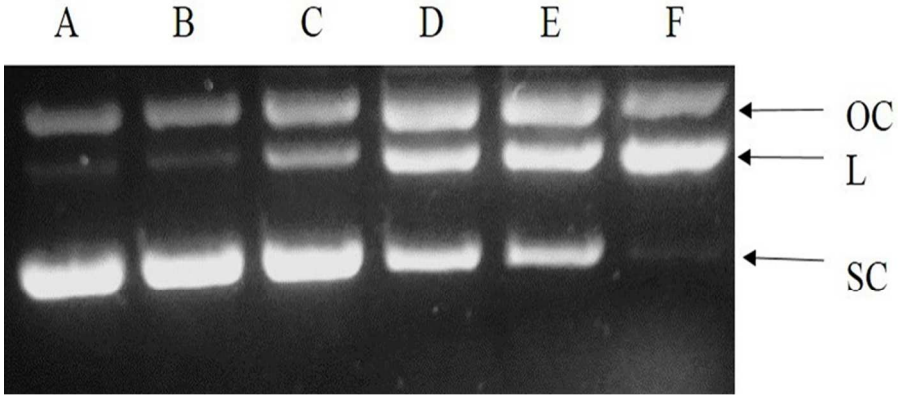

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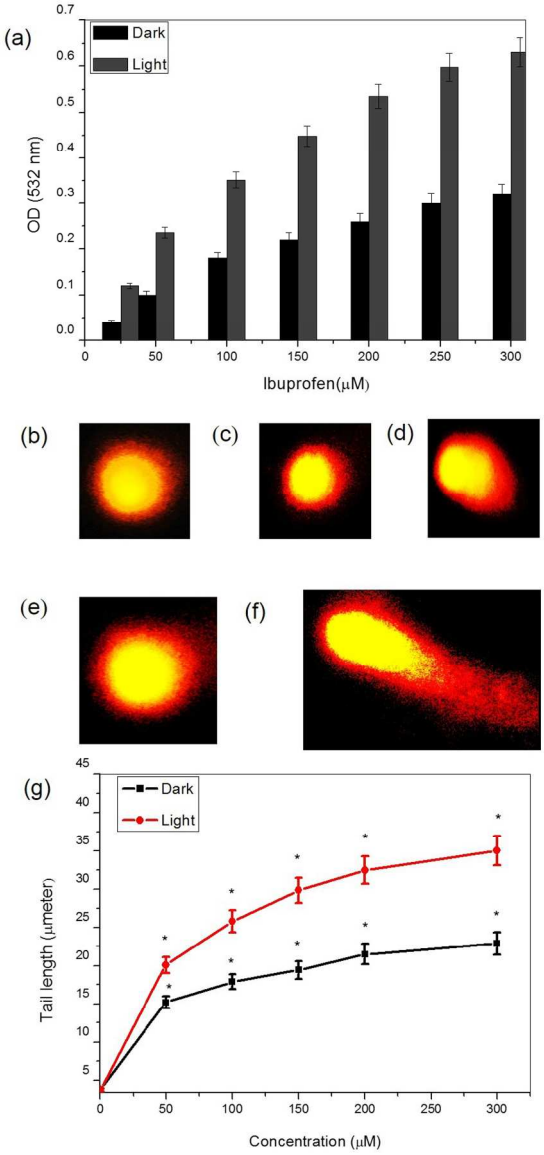

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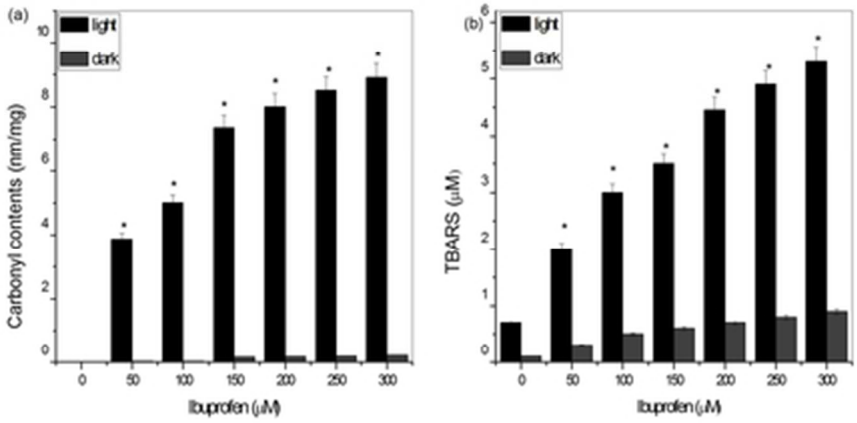

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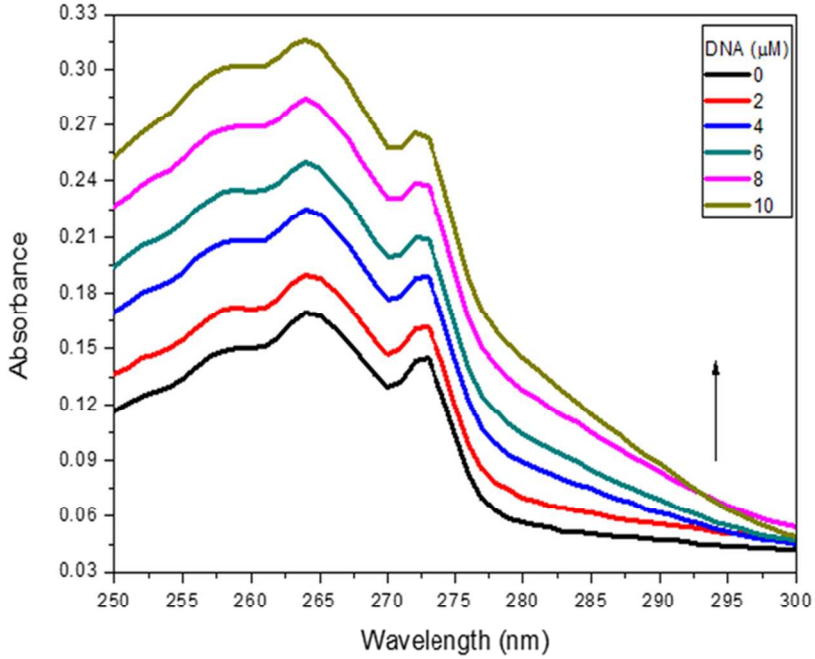

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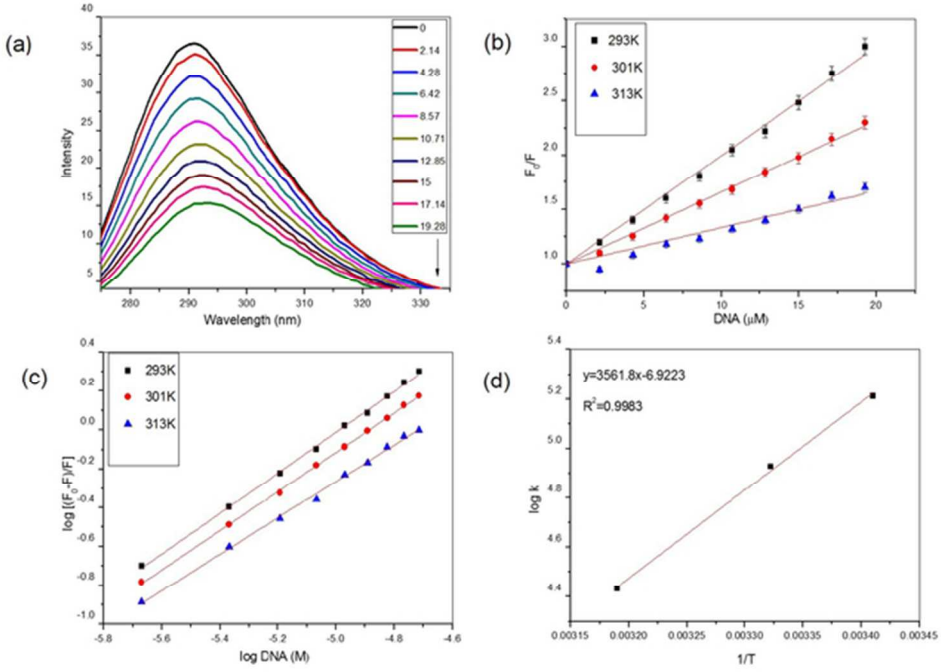

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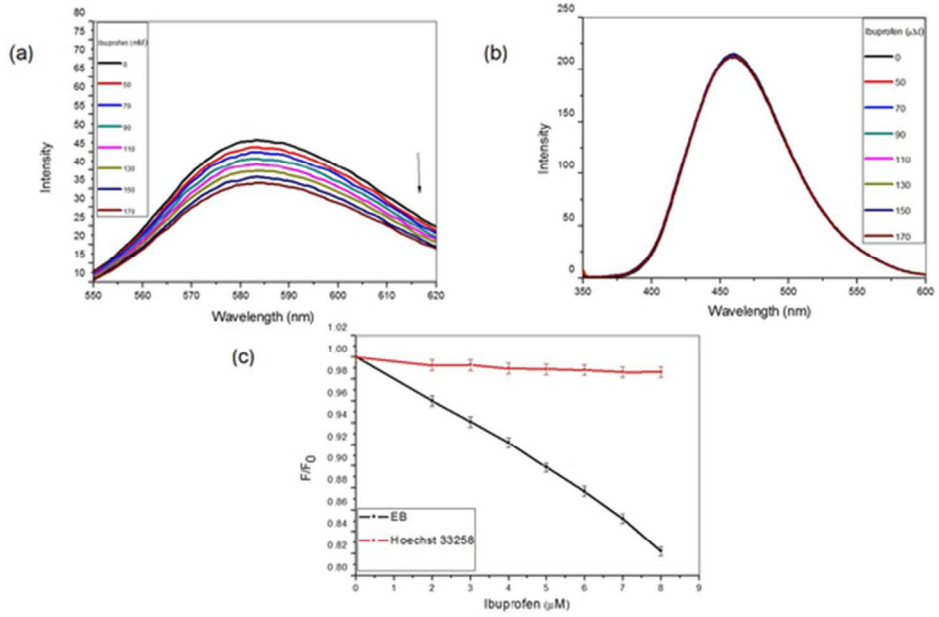

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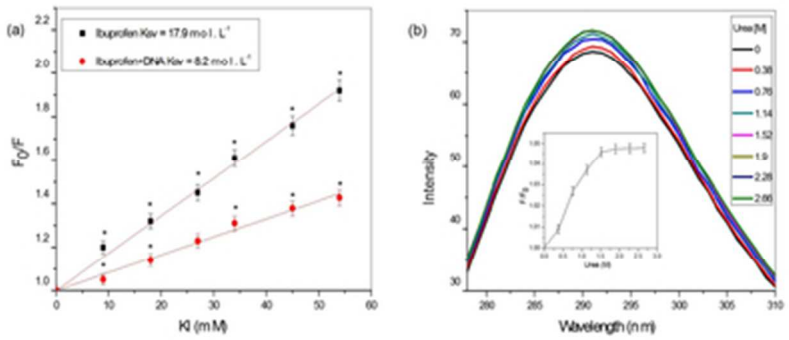

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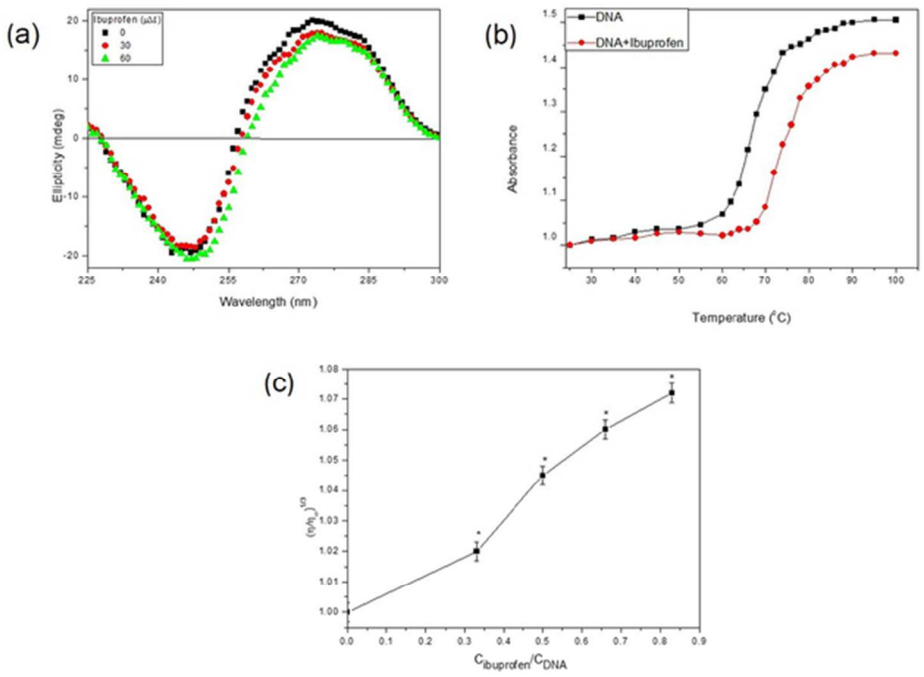
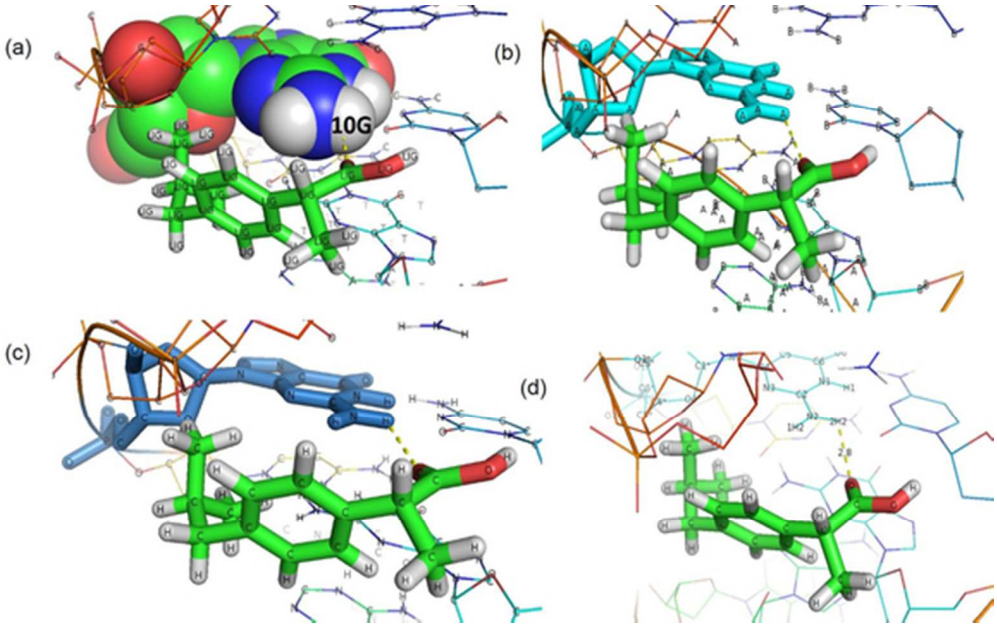
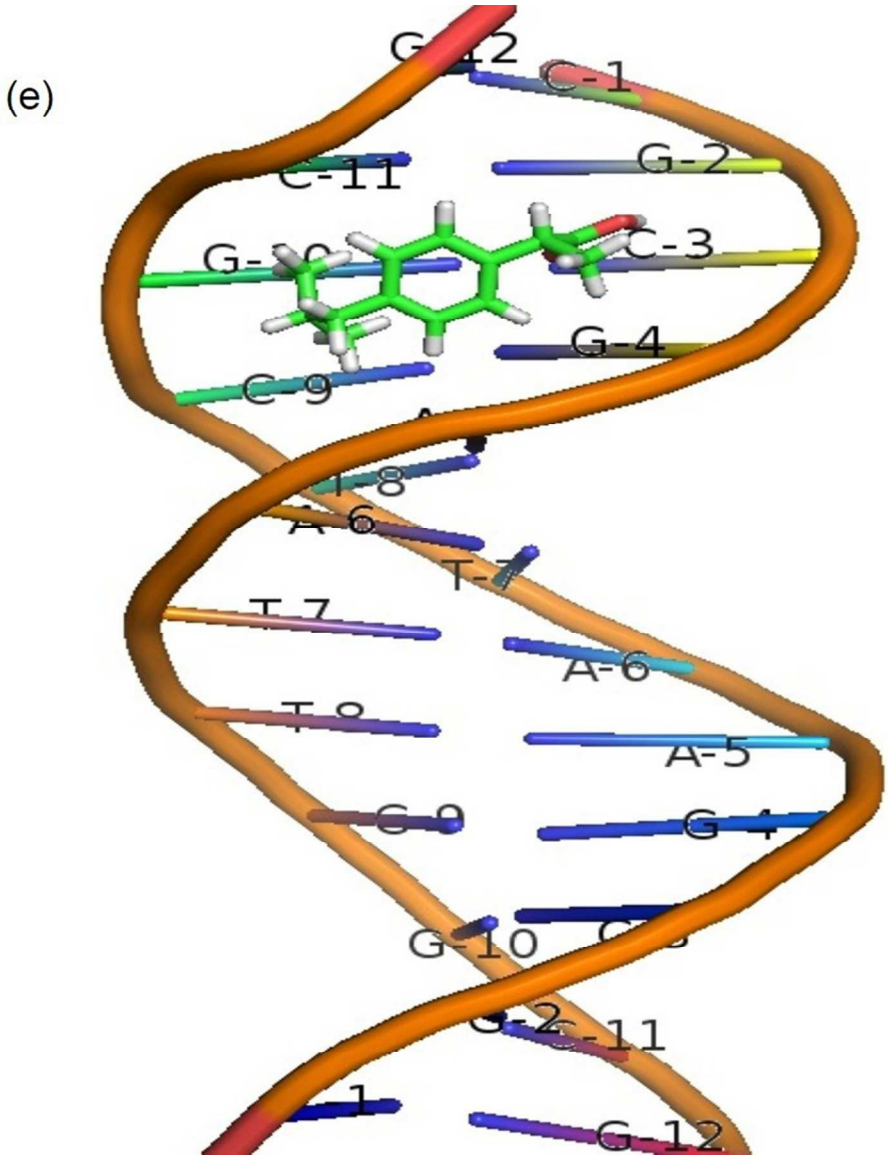


57x41mm (300 x 300 DPI)



49x30mm (300 x 300 DPI)



91x105mm (300 x 300 DPI)

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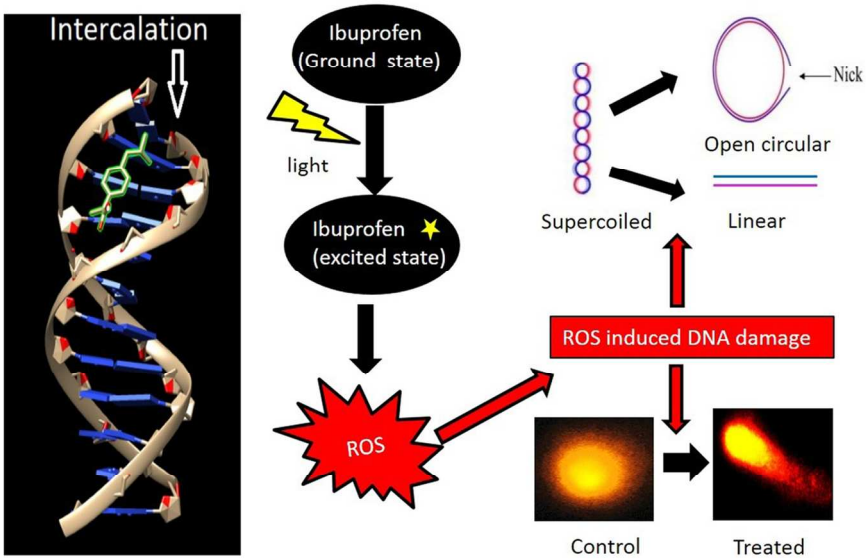
Ibuprofen causes photocleavage through ROS generation and intercalates with DNA: A combined spectroscopic and molecular docking approach



65x52mm (600 x 600 DPI)