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Molecular mechanisms of photosensitization XIII: a combined differential scanning calorimetry and DNA photosensitization study in non steroidal antiinflammatory drugs–DNA interaction

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Abstract

A combined differential scanning calorimetry (DSC) and photosensitization study has been carried out on the interaction of several NSAID on DNA, both from calf thymus and pBR 322 plasmid. The investigated compounds were both non-steroidal anti-inflammatory drugs as well as compounds related to NSAIDs for structural similar properties, to find evidence for their ability to interact with DNA as a function of steric hindrance and polarity of the chemical structures. The considered NSAIDs were diflunisal (DFN, a salicylic derivative), naproxen (NAP), ketoprofen (KPF), suprofen (SPF) and tiaprofenic acid (TIA, arylpropionic acids). The structural criterion used was related to three different aromatic groups, biphenyl, naphthalene and benzophenone (BZP). In fact drug–DNA interaction can be revealed by variations of the enthalpies and temperatures of unfolding of DNA obtained by comparison of calorimetric peaks, where a decrease of the enthalpy is associated with the drug–DNA interaction, by engaging electrostatic bonds. Testing their ability in inducing DNA cleavage when UVA irradiated can evidence the photosensitizing properties of the drug. A good correlation was found between calorimetric and photosensitization studies. From the results obtained it can be reasonably supposed that the photocleavage depends only on the drug molecules bound to DNA.

Keywords: Antiinflammatory; DNA; Differential scanning calorimetry; Photosensitization; NSAID

1. Introduction

The differential scanning calorimetry (DSC) is a technique able to investigate thermal processes (reversible, as the lysozyme unfolding, or irreversible as the protein denaturation) allowing to evidence now if the presence of drugs can influence their thermal behaviour. For instance, the thermal denaturation of DNA (calf thymus) has been found to be modified by the urea presence resulting in a shift of the calorimetric peak to higher temperatures, meaning that the urea is able to stabilize the DNA structure (Liu and Tan,

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1995). The thermal behaviour of DNA is affected by the presence of pair of basis Guanine–Cytosine, which causes an increase in the enthalpy of the transition by the presence of hydrogen bonds stabilizing the double helix (Wada et al., 1980). This behaviour appears to be very complex by the presence of a multipeak structure and the existence of a heterogeneity intermolecular forming a DNA satellite (Klump and Herzog, 1984; Maeda et al., 1984; Breslauer, 1986; Filimonov, 1986; Wada and Suyama, 1986; Klump, 1988). On the other hand, the interaction of several classes of drugs (antibiotics and antracicline (Maeda et al., 1990) and antitumoral drugs (Krugh, 1994)) have been reported in literature suggesting different way to exert the effects on the denaturation of DNA.

In this paper a combined DSC and photoinduced cleavage study has been carried out on the interaction of several NSAIDs on DNA, unsonicated calf thymus and on pBR322 plasmide. The investigated compounds reported here were both non-steroidal anti-inflammatory drugs as well as compounds related to NSAID for structural similar properties to find evidences for their capacity to interact with DNA in the steric hindrance and in the polarity of the chemical structures.

In fact the happened interaction of a drug with DNA can be revealed by variations of the enthalpies and temperatures of unfolding of DNA obtained by comparison of calorimetric peaks (Breslauer et al., 1986). This was observed in the study of the interaction of DAPI/DNA (Manzini et al., 1983) where a decrease of the enthalpy is associated with the intercalation of the drug in the DNA, by engaging electrostatic bounds.

The study of photosensitized DNA damage is needed for a deep understanding of both phototoxicity, as well as photoimmunology. Of course, independently on the primary photochemical act, the light induced side-effect needs to be more deeply qualified (for example, as phototoxic or photoallergic), and a more detailed investigation is requested. Thus, such a specific knowledge of the photobiological consequences in vivo, such as degenerative skin diseases, can help in attributing the photogenotoxic, photoimmunotoxic, or photohepatotoxic character of the event (Beijersbergen van Henegouwen, 1998; Gould et al., 1995). Moreover, it contributes to clarify the mechanisms of the cell toxicity of potential anticancer drugs. Under this respect it is very important to consider the biodistribution of the photosensitizing agent and its binding mode to DNA (Pyle et al., 1989; Kessel and Woodburn, 1993; Krugh, 1994; Condorelli et al., 1995). In fact the photosensitizer can associate to DNA either through a surface interaction such as hydrogen bonding or Van Der Waals forces along the grooves of the helix or through non-covalent intercalation via π-stacking of aromatic heterocyclic groups between base pairs. In the latter case, a site-selective photocleavage can be promoted. The study of the influence of parameters like hydrophobicity, geometry, size, shape and ability in the formation of H-bonds with the base pairs permits the assessment of the affinity between the photosensitizer and DNA. Useful information about modes of interaction is provided from several experimental strategies, such as induced linear and circular dichroism, fluorescence anisotropy measurements and low temperature phosphorescence (Jansen et al., 1993) as well as by the previously cited microcalorimetric technique.

The mechanism of photoinduced DNA damage, leading to nucleic acid oxidation and single strand break, involves three main pathways: (i) participation of hydroxyl radicals, known as one of the most noxious species in promoting DNA damage (Paillous and Vicendo, 1993); (ii) electron transfer (Kelly et al., 1987); (iii) oxidation via singlet oxygen (Piette, 1991). The photosensitization process efficiency is determined by measuring supercoiled plasmid linearization rates. This is obtained by monitoring the changes in conformation of supercoiled form I of DNA, which is at first converted in relaxed open circular Form II via single strand break and finally to linear Form III through double strand break (Artuso et al., 1991; Condorelli et al., 1995). DNA forms are isolated via agarose gel electrophoresis (see Section 2).

The NSAIDs drugs selected for this study were diflunisal (DFN), naproxen (NAP), suprofen (SPF), tiaprofenic acid (TIA) and ketoprofen (KPF). All these drugs were reported in clinical

cases of phototoxic (Ljunggren, 1985 and literature there cited) and photoallergic reactions (Valsecchi et al., 1989; Suarez et al., 1990; Tosti et al., 1990; Kurumaji et al., 1991) and the molecular mechanism of photosensitization 'in vitro' have been widely studied (Moore and Chappuis, 1988; Costanzo et al., 1989a,b; Boscà et al., 1990, 1994; De Guidi et al., 1991, 1997; Castell et al., 1993; Condorelli et al., 1996; Monti et al., 1997; Sortino et al., 1998). The reason why many NSAIDs are able to induce photosensitization lies firstly in their ability to absorb the radiation available in the environment, which is able to penetrate skin (at wavelengths longer than 310 nm) and this of course is related to their chemical structure. Thus an in vitro device able to mimicking a photosensitization system can provide useful information for the overall problem. Of course, it must be taken in account that after the 'photosensitizing act', i.e. absorption of natural occurring light by an exogenous agent and production of stable and transient species able to interact with biosubstrates, the sequence of events correlated either to a phototoxic or a photoallergic phenomenon does not fall in the aims of this paper.

2. Materials and methods

2.1. Materials

DFN, NAP, KPF, SPF, 2-methoxynaphtalene, naphtalene and benzophenone (BZP) were obtained from Sigma–Aldrich (Italian division, Milan. Italy), TIA was a gift of Erregierre S.P.A. (Milano, Italy), all other chemicals were reagent grade. Phosphate buffer saline (PBS) (pH 7.4) consisted of a 10 mM phosphate buffer solution added with 5×10^{-2} M NaCl. Supercoiled pBR322 DNA (sc-DNA), Form I, molecular weight (MW) 2.9×10^6 Da, 4365 base pairs (bp) and calf thymus DNA (ct-DNA) were obtained from Pharmacia (Milano, Italy). The percentage of relaxed Form II of pBR322 was less than 12% in the starting material and no linear Form III was detected (the data were obtained from densitometric analysis of agarose gel electrophoresis). All preparations were made by using Millipore bidistilled water.

2.2. Irradiation conditions

In all the photosensitization experiments the irradiation was performed using a Rayonet photochemical reactor equipped with 8 'black light' phosphor lamps with an emission in the 310–390 nm range with a maximum at 350 nm. The fluency rate at the irradiation position was about 800 $\mu W/cm^2$. The incident photon flux on either 3 ml solution in quartz cuvettes (optical length, 1 cm) (photohemolysis and photoperoxidation experiments) or on a 18 μl solution in the Eppendorf tubes (0.2 cm of optical path) (DNA experiments), was 6×10^{16} and 2.8×10^{15} quanta/s respectively. A 'merry-go-round' irradiation apparatus was used to ensure that all the samples received equal radiation. (Irradiation times ranged between 30 and 60 min, depending on the compound used, on its fraction of absorbed light and DNA photocleavage quantum yield, so to maintain comparable the cleavage rates.)

The experimental procedures of irradiation and the light intensity measurements have been described previously (De Guidi et al., 1994; Condorelli et al., 1995).

2.3. DNA photocleavage experiments

The samples containing pBR322 DNA and sensitizer were prepared in a final volume of 18 μl, placed in Eppendorf tubes and irradiated in a Rayonet photochemical reactor (see Section 2.2). Following irradiation, 4 μl of a mixture composed of 0.22% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1 mM EDTA pH 8 and sodium lauryl sulphate (0.5% w/V), were added to the samples. A total of 18 μl of each sample (0.4 μg DNA) were loaded onto 1% agarose gel of 5 mm thickness (up to 22 wells). The electrophoretic analysis was performed in tris–borate–EDTA buffer with a Pharmacia horizontal apparatus (GNA-200). The power supply was set at 40 V for 15 h at 25°C. Following electrophoresis, the gel was stained with ethidium bromide (1 μg/ml) for 30 min and rinsed with a $MgCl_2$ solution (10 mM) for 20 min.

DNA forms were detected by excitation of ethidium bromide fluorescence on a 300 nm UV

transilluminator (Pharmacia). Photographs were taken with a Nikon F50 camera equipped with a 60 mm AF Micro lens Nikkor and a red filter. Quantitation of bands was achieved by microdensitometry of the negative produced from the gel photograph using a Beckman DU 650 spectrophotometer equipped with gel scan accessory.

The fraction of sc-DNA after electrophoresis was calculated through the following equation:

$$\text{sc}-\text{DNA} = \frac{\text{Area}_{\text{sc}}}{\text{Area}_{\text{sc}} + \sum \text{Area}_{\text{cl}}/1.66}$$

where Area_{sc} and Area_{cl} are respectively the percentages of sc-DNA and cleaved DNA (Forms II and III) which are both detected through densitometric analysis of fluorescent gels. The correct proportions of Forms I, II and III in each sample were calculated by using the coefficient 1.66 for the lower efficiency of ethidium bromide in binding to the Form I DNA with respect to Form II and III (Ciulla et al., 1989). In the photocleavage experiments no more than 10% Form III DNA was produced.

2.4. DFN photoproduct isolation

The main DFN photoproduct, the 2′-(2‴,4‴-difluoro-3″-carboxy-[1″,1‴-biphenyl]-4″-oxy)

× -4′-fluoro-4-hydroxy-[1,1′-biphenyl]

× -3-carboxylic acid, was prepared according to the literature by irradiating DFN PBS solution, following extraction with chloroform and subsequent separation on silica gel column using benzene–acetic acid–methanol (45:4:2.5, v/v/v) as eluent. Other details have been described previously (De Guidi et al., 1991).

2.5. Samples preparation for DSC measurements

A fixed amount of DNA and different amounts of drug, so to obtain increasing drug molar ratio (R) in respect to DNA, ranging from 0 to 1, were weighed inside a 150 μl DSC aluminium crucible. The samples were added with a fixed amount (110 μl) of SIT buffer (50 mM sodium chloride and 20 mM phosphate) at pH 7.4, and a 3×2 mm diameter Teflon coated stirrer magnet was inserted inside the DSC pan. The aluminium crucibles were hermetically sealed by a press and left on a stirrer for 1 h at 20°C and 12 h at 7°C. By using this procedure will be allowed a complete hydration of DNA and a drug dissolution, penetration and eventual interaction with DNA.

2.6. DSC

DSC measurements were carried out in a TA-4000 Mettler apparatus, equipped with a DSC 30 calorimetric cell and a TC-11 processor. The calorimetric cell was constituted by an Au/Au–Ni multipoint sensor inserted in a furnace temperature-controlled by a nitrogen flux coming from a liquid nitrogen heater. As reference sample a DSC pan filled with SIT buffer and a mini Teflon stirrer was employed. The samples were submitted to a heating scan between 30 and 120°C. The heating rate was 4°C/min and the sensitivity was 1.5 mW. Using indium, stearic acid and water carried out calibration temperature of the calorimetric system. Indium was employed to calibrate the transitional enthalpies (ΔH). Temperature and enthalpies were checked also by using palmitic acid. Enthalpies were evaluated from the peak areas using the integration program of the TA processor, permitting to choose different baselines and ranges of integration. The areas calculated with these different methods lie within the experimental error ($\pm$ 5%).

The denaturation temperatures, obtained by the calorimetric curves of the pure DNA as well as for drug loaded DNA, were reported as $(\Delta T/T_{\text{d}}^0)$ so to minimize eventual small differences in the denaturation temperatures from different DNA stocks. $\Delta T = T_{\text{d}} - T_{\text{d}}^0$, where T_{d} represents the denaturation temperature of DNA in presence of drug and T_{d}^0 is the denaturation temperature of pure DNA.

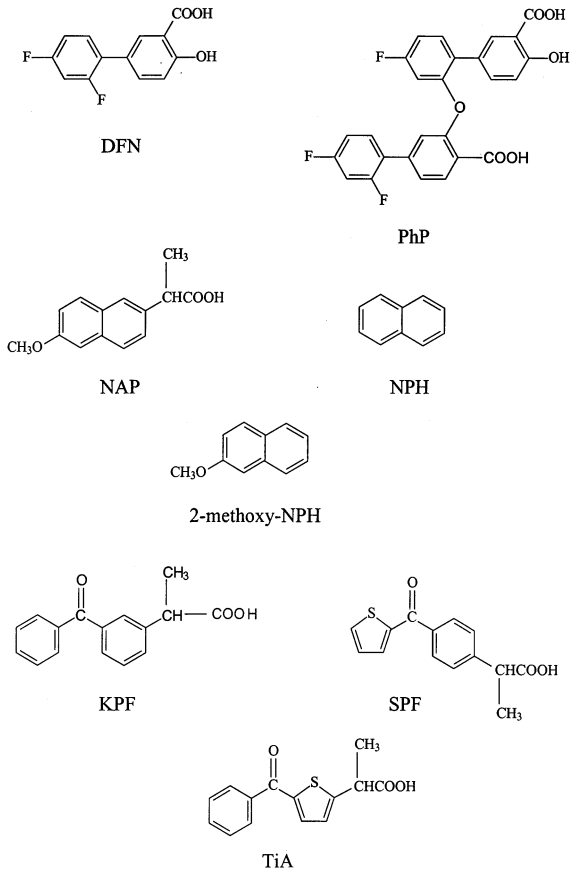
3. Results and discussion

The investigated drugs DFN, NAP, SPF, TIA and KPF were chosen on the basis of a structural criterion: they are all carboxylic acids: the former is a salicylic derivative, the others present a propi-

onic unit. Moreover, the aromatic moieties consist of classic chromophores with a well-known photochemistry and photophysics: biphenyl (for DFN), naphtalene (for NAP) and diarylketone (for KPF, SPF and TIA). The chemical structures are represented in Scheme 1.

3.1. DFN, DFN photoproduct–DNA interactions

DFN, the 2′,4′-difluoro-4-hydroxy-[1,1′-byphenyl]-3-carboxylic acid, presents a biphenyl group. This drug was found to be quite phototoxic 'in vivo', as tested in the mouse tail assay in sub-lethal doses (Ljunggren, 1985). Defluorination was found to be the primary photochemical act, leading to the photosensitization processes; the noxious species involved were the consequently formed free radicals, superoxide anion, singlet



Scheme 1. Chemical structures of the investigated molecules.

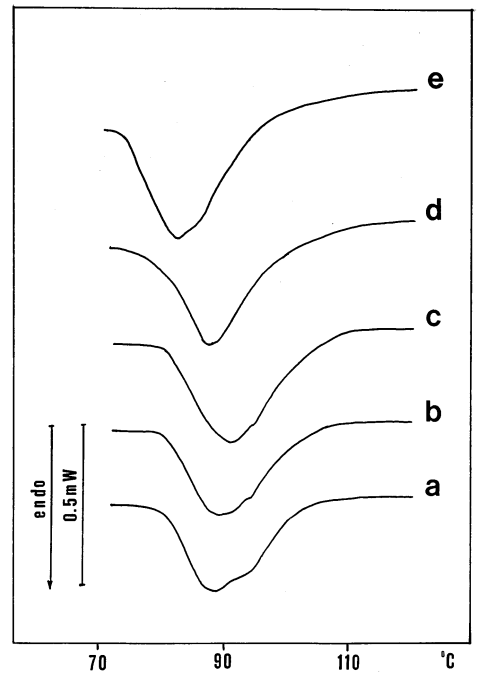


Fig. 1. Typical calorimetric curves for calf-thymus-DNA (ct-DNA) denaturation [1.9×10^{-2} M] in presence of diflunisal (DFN) at the following ratios ($R = [\text{DFN}]/[\text{DNA}_{bp}]$): $a = 0$; $b = 0.125$; $c = 0.250$; $d = 0.50$; $e = 1.0$.

oxygen and, mostly, the main photoproduct, the 2′-(2‴,4‴-difluoro-3″-carboxy-[1″,1‴-biphenyl]

× -4″-oxy)-4′-fluoro-4-hydroxy-[1,1′-biphenyl]

× -3-carboxylic acid (PhP), a compound formed through $S_{RN}1$ which is a typical reaction for aryl-halides (De Guidi et al., 1991).

The calorimetric curves of DNA denaturation in the presence of increasing amounts of DFN are reported in Fig. 1. It appears evident how the drug, by interacting with DNA, modifies the calorimetric curve of the pure DNA by shifting the denaturation peak initially at higher values (until a DFN/DNA ratio $R = 0.25$) then decreasing the transitional temperature. A value approximately constant is observable for the associate enthalpy variation (ΔH). These results are summarized also in Table 1 where the T_d (°C), $\Delta T/T_d^0$, (being $\Delta T = T_d - T_d^0$ the difference, respectively, between the denaturation temperature in presence

of drug and of pure DNA) and ΔH (J/g) are reported.

This behaviour should suggest the existence of different kind of interactions DFN–DNA related to the drug concentration. Interactions of the drug, at lower concentrations, can be supposed in an approaching to the helix by a cooperative mechanism (Kochevar, 1989). In this case, when a certain number of molecules of the sensitizer bind to DNA, alters the double helix structure, thus favouring the interaction of the next molecules in the same domain. This could also explain the trend of the calorimetric curves.

DFN induces DNA photocleavage; if the dependency of the damage percentage on the [DFN]/[DNA_{bp}] (in base pairs) ratio (R) is observed, it can be noted that the plateau shown in Fig. 2 is reached at a sensitizer concentration at which the incident light is not completely absorbed. This suggests that DFN associates to DNA in a concentration depending fashion and that only bound molecules are responsible for the cleavage.

The main DFN photoproduct, the 2′-(2‴,4‴-difluoro-3″-carboxy-[1″,1‴-biphenyl]-4″-oxy)

× -4′-fluoro-4-hydroxy-[1,1′-biphenyl]

× -3-carboxylic acid (PhP), which has been isolated according to literature (De Guidi et al., 1991) has been found to be the real photocleaving agent (Fig. 3) (Costanzo et al., 1996) without displaying any thermal action, contrarily to what found for membranes (De Guidi et al., 1991). For this reason, is is considered important to investigate the interaction of this compound with the double helix. The results of PhP–DNA interaction, studied by calorimetric measurements, are reported in Table 1. It appears evident the compound ability to interact by causing a T_d shift toward lower values, with respect to pure DNA, with a concomitant ΔH decrease.

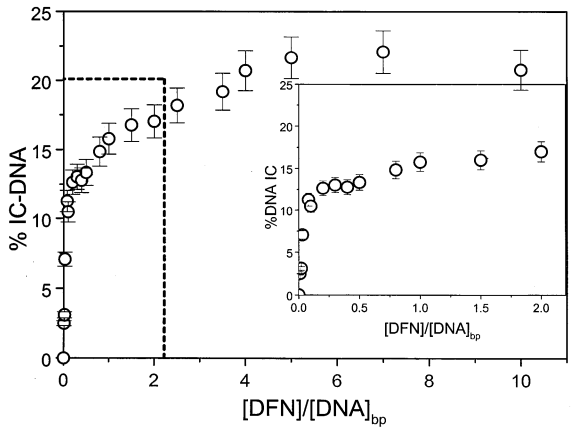


Fig. 2. pBR322 DNA (33 μM bp) induced cleavage (IC) vs [DFN]/[DNA_{bp}] ratio (R) in aerobic conditions, in phosphate buffer saline (PBS) solution at 25°C. Irradiation time 60 min. Each point represents the mean of three experiments (S.D. = 10%).

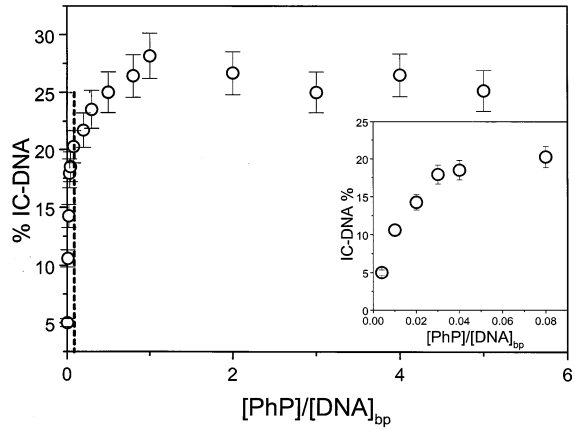


Fig. 3. pBR322 DNA (33 μM bp) induced cleavage (IC) vs [PhP]/[DNA_{bp}] ratio (R) in aerobic conditions, in phosphate buffer saline (PBS) solution at 25°C. Irradiation time 60 min. Each point represents the mean of three experiments (S.D. = 10%).

These results are in opposition to those observed for DFN (initial T_d shift at higher values), but confirm the hypothesis that the PhP, formed during DFN irradiation, should be the main cause of the DNA degradation. In fact it should be taken into account that PhP, produced by irradiation of DFN (free or bound), is able to substitute to the double helix bound DFN molecules, which have a stabilizing effect associate to a lower photosensitizing efficiency. Thus, this further evidences the close interaction between the molecular interaction and photoinduced cleavage. As a consequence it can be reasonably supposed that DNA light induced damage depends exclusively by DNA-bound drug molecules.

Table 1
Denaturation temperature (T_d, °C), the related $(\Delta T/T_d^0)\times 10^2$, and the enthalpic changes of denaturation process (ΔH, J/g) for studied compounds[a]

[Drug]/[DNA_{bp}]	DFN			PhP(DFN)								
	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)						
0	(89.3)	0.0	40.5	(90.5)	0.0	37.5						
0.125	(89.3)	0.0	40.4	(86.7)	−4.2	27.8						
0.25	(93.0)	4.1	43.1	(86.9)	−4.0	29.0						
0.50	(89.3)	0.0	39.2									
1.0	(83.4)	−16	41.1									
[Drug]/[DNA_{bp}]	NAP			2-Methoxy-NPH			NPH					
	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)			
0	(89.5)	0.0	40.0	(89.5)	0.0	40.9	(90.2)	0.0	50.0			
0.125	(88.6)	−1.0	38.1	(90.5)	1.1	38.2	(88.8)	−1.5	52.3			
0.25	(86.1)	−3.8	37.8	(91.3)	2.0	37.0	(90.2)	0.0	47.0			
0.50	(87.0)	−2.8	33.4	(90.4)	1.0	39.5	(87.9)	−2.5	40.1			
1.0	(85.0)	−5.0	34.9	(90.1)	0.7	35.5	(87.7)	−2.8	61.5			
[Drug]/[DNA_{bp}]	KPF			SPF			TIA			BZP		
	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)	(T_d)	$(\Delta T/T_d^0)\times 10^2$	ΔH (J/g)
0	(89.7)	0.0	42.8	(89.7)	0.0	38.5	(90.1)	0.0	48.9	(90.6)	0.0	42.8
0.125	(89.3)	−0.6	38.7	(89.5)	−0.2	36.5	(89.5)	−0.7	45.0	(90.0)	−0.7	44.1
0.25	(88.9)	−0.9	31.9	(89.5)	−0.2	33.8	(89.1)	−1.1	47.9	(89.6)	−1.1	45.0
0.50	(87.9)	−2.0	37.8	(88.4)	−1.4	35.0	(87.3)	−3.1	43.1	(90.6)	0.0	43.3
1.0	(85.6)	−4.6	39.0	(86.8)	−3.2	33.1	(78.2)	−13.2	42.0	(90.5)	−0.1	45.1

[a] Data are obtained from at least three measurements.

3.2. NAP, 2-methoxy-NPH, NPH–DNA interactions

NAP (6-methoxy-α-methyl-2-naphtalene acetic acid) like KPF, SPF, and TIA, belongs to the chemical class of the arylpropionic acids. Under UVA irradiation, these drugs undergo decarboxylation as the main photochemical process in aqueous or methanolic solutions (Condorelli et al., 1996; De Guidi et al., 1997). Subsequent thermal steps lead to production of several photoproducts, some of which display toxic and/or phototoxic properties. Transients like free radicals, oxygen activated species and solvated electrons have been indicated to be potentially responsible for the observed photosensitizing effects. Hydrogen abstraction and oxidative steps are involved in these processes.

NAP is well known to cause bullous skin photosensitivity (Suarez et al., 1990) and its photochemical reactivity proceeds both via photoionization and triplet state production, leading to singlet oxygen formation (Moore and Chappuis, 1988; Costanzo et al., 1989a). NAP photolysis involves decarboxylation with production of free radicals; a new photodegradation pathway involving a carbenium ion, whose formation can be explained by homolysis of a radical cation after photoionization, was proposed by Boscà et al. (Boscà et al., 1990).

The calorimetric study of NAP–DNA interaction has been integrated with two other compounds, which presents structural analogies, the 2-methoxynaphtalene and the naphtalene.

The calorimetric curves of the NAP–DNA interaction are reported in Fig. 4. It appears evident how the denaturation temperature of the DNA is dependent on NAP concentration, decreasing by increasing the drug concentration. The associated enthalpy variation shows a decreasing trend until the attainment of a constant value. By increasing the drug concentration, a splitting of the curve in two components starts.

These data are in good agreement with DNA cleavage photoinduced by this drug. The ability of NAP to induce DNA photocleavage has been previously investigated (Artuso et al., 1991; Giuffrida et al., 1996). Photoinduced DNA strand break rate is enhanced in anaerobic conditions, though photodecarboxylation quantum yield shows an opposite behaviour. The drug photogenerated radicals and the hydroxyl radicals (produced by the decay of superoxide anion, a species originated during NAP photodegradation) were found to be the involved transient. Participation of singlet oxygen was ruled out and an energy transfer mechanism was also postulated (Artuso et al., 1991; Giuffrida et al., 1996).

In Fig. 5 the photoinduced cleavage is plotted as a function of drug/DNA ratio. As it can be noted, when the ratio reaches and passes the value of 0.5 a change in slope is observed and finally a plateau at $R \cong 1$ is obtained. Thus the following hypotheses can be reasonably sustained:

1. An interaction is operative between drug and DNA: it probably provokes a partial unwinding of the double helix and thus less energy is requested to obtain a complete denaturation.

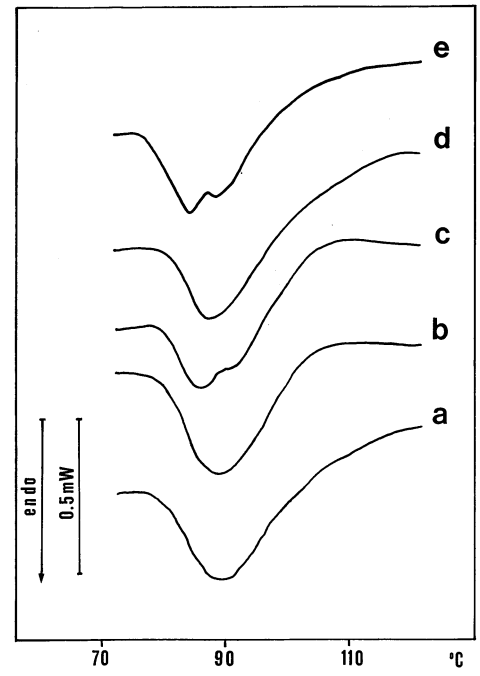


Fig. 4. Typical calorimetric curves for calf-thymus-DNA (ct-DNA) denaturation [1.9×10^{-2} M] in presence of naproxen (NAP) at the following ratios ($R = [NAP]/[DNA_{bp}]$): $a = 0$; $b = 0.125$; $c = 0.250$; $d = 0.50$; $e = 1.0$.

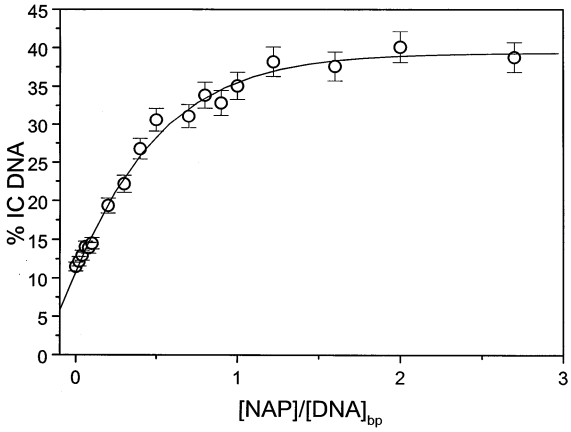


Fig. 5. pBR322 DNA (33 μM bp) induced cleavage (IC) vs [NAP]/[DNA_{bp}] ratio (R) in aerobic conditions, in phosphate buffer saline (PBS) solution at 25°C. Irradiation time 40 min. Each point represents the mean of three experiments (S.D. = 10%).

2. NAP binds to DNA until a ratio $\cong 0.5$. Further addition of drug does not cause strong changes either in the thermodynamic parameters or in photosensitized cleavage. So NAP excess remains in the bulk of solution.
3. DNA photoinduced cleavage is caused only by NAP bound molecules.

The effect of 2-methoxy-NPH on the melting temperature is poor (Table 1), in fact it remains nearly constant. Instead, it influences the shape of the calorimetric peak, which is affected by the fusion peak of the solid compound, which probably remains undissolved inside the DNA structure as well as in the aqueous medium. The presence of solid drug should be the reason of the raising at higher temperatures of a secondary peak, overlapped to the main DNA peak (calorimetric curves not shown).

The irradiation of 2-methoxy-NPH in the presence of DNA does not provokes any significant cleavage, thus strengthening the hypothesis that interaction is the key step for an efficient photoinduced cleavage.

The NPH is, instead, able to interact with DNA (Table 1) probably because of the polar groups absence (Sartorius and Schneider, 1997). This interaction causes a T_d shift towards lower values and smaller variations are observed as regards the ΔH. Like the 2-methoxy-NPH a secondary peak was observed at low drug concentrations. In addition DNA photosensitized cleavage by NPH is observed (Fig. 6). On the other hand, the efficiency of this process is not very high, as expected by a singlet oxygen-type II photosensitization mechanism, not mediated by photogenerated radicals (Piette, 1991).

3.3. KPF, SPF, TIA, BZF–DNA interactions

KPF, SFP and TIA are three diaryl–ketone–propionic acids; SPF differs from the BZP-like KPF by replacement of the non substituted benzene ring with a thiophene one and for the position of the propionic group in *para* instead of *meta*, whereas TIA shows inversion of the positions of the benzene and thiophene rings. For this reason their photochemistry is often closely related (Monti et al., 1998).

The 2-(3-benzoylphenyl) propionic acid (KPF), is one of the most common between the NSAIDs present in the market. It was shown to be able to elicit photocontact dermatitis in a number of cases (Suarez et al., 1990; Tosti et al., 1990). Its photodegradation has been investigated in buffered media and it was found that KPF undergoes a decarboxylation process with a high quan-

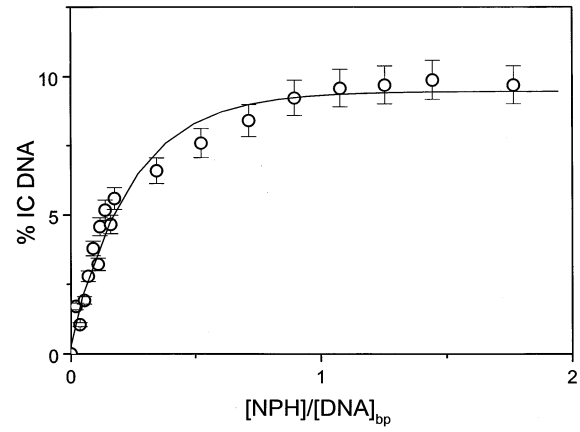


Fig. 6. pBR322 DNA (33 μM bp) induced cleavage (IC) vs [NPH]/[DNA] ratio (R) in aerobic conditions, in phosphate buffer saline (PBS) solution at 25°C. Irradiation time 40 min. Each point represents the mean of three experiments (S.D. = 10%).

tum yield (Costanzo et al., 1989a). Singlet oxygen is generated only by its photoproducts, whereas superoxide anion is produced in large amounts. Time resolved studies indicated that an intramolecular electron transfer in the triplet state is at the basis of the decarboxylation process (Monti et al., 1997). Due to the reactivity of the aromatic carbonyl moiety, minor photoproducts involving initial hydrogen abstraction by the excited BZP chromophore and/or dimerization have been detected (Boscà et al., 1994).

The photosensitivity 'in vivo' of SPF, (2-[4-(2-thenoyl)phenyl]propionic acid), was observed: this drug triggered photocontact dermatitis as shown by photopatch tests (Kurumaji et al., 1991). Photochemical and photophysical deactivation of the SPF lowest triplet excited state responsible for decarboxylation, having a π-π* thiophenic nature, was studied through time resolved emission and absorption spectroscopy (Monti et al., 1998; Sortino et al., 1998).

The phototoxicity of TIA, the (2-[4-(2-benzoyl)thiophenyl]propionic acid), has been clinically proven with the appearance of side effects such as erythema flaring and urticarial weal (Valsecchi et al., 1989). Photodegradation of TIA in aqueous solutions leads via excited triplet state to decarboxylated products with ethyl, 1-hydroxyethyl and/or acetyl side chain (Castell et al., 1993; Encinas et al., 1998; Monti et al., 1998). Peroxidic intermediates are presumably involved under aerobic conditions.

The calorimetric investigation of these compounds interaction with DNA results, respectively, in:

1. a shift of the calorimetric curves and of the associated T_d toward lower values with a poor variation in the enthalpy change, indicating that the KPF makes the process easier by lowering the denaturation temperature providing enough energy to leave the process unvaried (Table 1);
2. the SPF behaviour is similar to the KPF with a less evident shift, suggesting a minor interaction with respect to KPF (Table 1);
3. the TIA effect is stronger than the other examined samples only for high concentrations values ($R = 1$), instead at lower values in similar (Fig. 7 and Table 1).

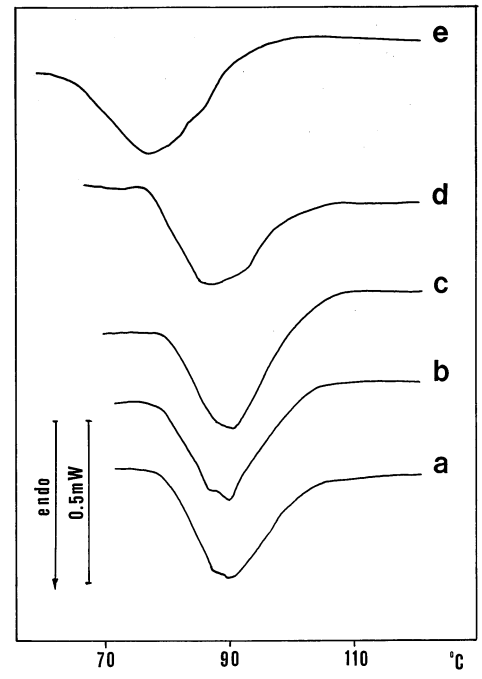


Fig. 7. Typical calorimetric curves for calf-thymus-DNA (ct-DNA) denaturation [1.9×10^{-2} M] in presence of tiaprofenic acid (TIA) at the following ratios ($R = [TIA]/[DNA_{bp}]$): $a = 0$; $b = 0.125$; $c = 0.250$; $d = 0.50$; $e = 1.0$.

By these results, it was worthy to extend the investigation to the BZP. In this compound, possessing a structural feature close to those exhibited from the examined drugs, the propionic group, which can be considered as the major cause of the drug interaction with DNA, is missing. It appears evident, by looking to Table 1, the lacking of any interaction of this compound with DNA, in fact, neither T_d or ΔH variations are observable.

Between the three drugs, only SPF photosensitization on DNA has been studied in detail (Condorelli et al., 1995). A photosensitization mechanism involving singlet oxygen and free radicals has been proposed. The higher rate of photocleavage in nitrogen compared to that in air saturated solution and the results obtained from oxygen consumption measurements support the following hypothesis: (a) that both type I and type II photosensitization mechanisms are operative; (b) that oxygen quenches the excited state of the

irradiated drug, according to Sortino et al. (1998). The photosensitization model applied is in agreement with that applied to cell membrane SPF photoinduced damage. Interaction of the drug with DNA, studied through circular dichroism and fluorescence anisotropy, probably occurs through surface binding mode (De Guidi et al., 1994).

If the photosensitizing activities of the three drugs are compared (Fig. 8), it can be affirmed that at least at low ratios, they follow the trend of interaction with DNA, deduced by the calorimetric curves. On the other hand, it must be taken into account: (a) the different mechanisms involved in the photosensitization process (type I free radicals and type II singlet oxygen); (b) the drug photodegradation quantum yields; (c) the potential photosensitizing activities of the drug photodegradation products. Thus care has to be taken in a strictly quantitative comparison of the DNA damaging potency.

The analysis of the BZP–DNA photocleavage (Fig. 9) provides interesting considerations. In fact, BZP shows a well known photochemistry of hydrogen abstraction, starting from excitation of n–π* band of aromatic carbonyl with consequent sensitized peroxidation in lipid membrane (Markovic and Patterson, 1993). Despite this fact,

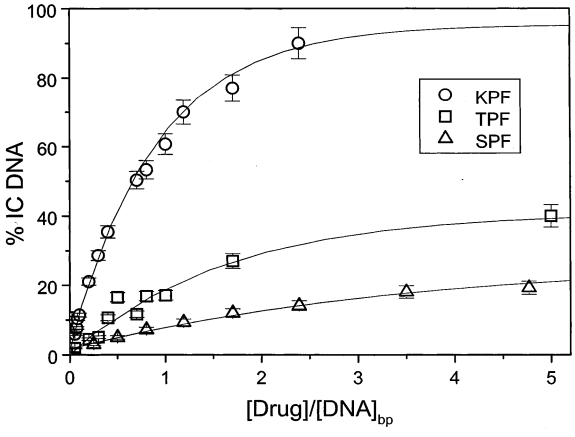


Fig. 8. pBR322 DNA (33 μM bp) induced cleavage (IC) vs [KPF, TIA, SPF]/[DNA] ratio (*R*) in aerobic conditions, in phosphate buffer saline (PBS) solution at 25°C. Irradiation time 30 min. Each point represents the mean of three experiments (S.D. = 10%).

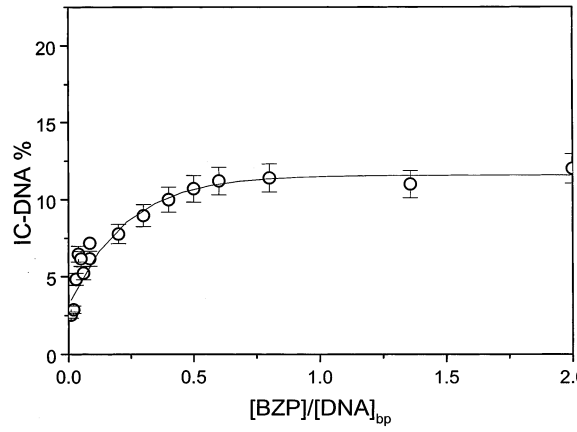


Fig. 9. pBR322 DNA (33 μM bp) induced cleavage (IC) vs [BZP]/[DNA] ratio (*R*) in aerobic conditions, in phosphate buffer saline (PBS) solution at 25°C. Irradiation time 30 min. Each point represents the mean of three experiments (S.D. = 10%).

it shows low DNA cleavage efficiency, confirming that interaction is a limiting conditions for DNA-cleaving activity. This is confirmed by the fact that NPH, which is a photosensitizer potentially less active than BZP, shows efficiency comparable to this latter, due to its ability in interacting with DNA.

4. Conclusions

The aim of this research was mainly to see how two techniques, DSC and photosensitization on biological systems (Photocleavage) which are deeply different from each other, can find application in trying to clarify the interaction mechanisms between pharmacologically active compounds, or their derivatives, and biological substrates like the DNA used by us. The experimental results confirmed how the two techniques give suggestions on the drug–DNA interactions, which are in agreement with the literature and can be very useful in the understanding of the interaction mechanisms. These results can find application in a subsequent research step, when the natural sequence interaction-photosensitization-biological consequences could be analyzed in a inter-disciplinary approach.

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