

Interaction of a Potential Anticancer Agent Hypericin and its Model Compound Emodin with DNA and Bovine Serum Albumin

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Abstract. *Background/Aim:* We report the incorporation of prospective anticancer agent hypericin into DNA and bovine serum albumin (BSA), respectively, with emphasis on comparison of the differences in interaction mode between hypericin and its model compound emodin. *Materials and Methods:* Spectrophotometric methods were used for determination of the binding constants of the drug complex with biomacromolecules. Differential scanning calorimetry was applied for evaluation of drug-macromolecule complex thermal stability. *Results:* The strength of interaction expressed by binding constants was found to be 4.0×10^4 l/mol for hypericin–DNA and 8.1×10^4 l/mol for emodin–DNA complex. Both molecules stabilize bovine serum albumin macromolecule and bind into the hydrophobic cavity in IIA subunit but their localization within the molecule is different. *Conclusion:* Anticancer agent hypericin and its derivative emodin interact with DNA with medium strength and are probably incorporated into the groove of DNA by hydrogen bonds. Bovine serum albumin can serve as a transport protein for hypericin since the binding force between both molecules is adequate.

Hypericin (4,5,7,4',5',7'-hexahydroxy-2,2'-dimethylnaphthodiantrone) is a natural photosensitizing pigment occurring in plants of the genus *Hypericum*. Hypericin (Figure 1A) under light illumination displays antiproliferative and cytotoxic effects on many tumor cell lines (1, 2). These properties together with minimal dark toxicity, tumor selectivity and high

clearance from the host body make hypericin a very promising agent in photodynamic therapy of cancer. Due to its prospective pharmaceutical utilization, this interesting molecule has been the subject of many scientific research studies (3-6). Model compound of hypericin, emodin (3-methyl-1,6,8-3hydroxy-anthraquinone, Figure 1B) was considered to our study for its structural similarity to the hypericin molecule. In addition, emodin, a plant-derived anthraquinone, was found to be a photosensitizer which possess antitumor, antibacterial, antiviral, anti-inflammatory, and myorelaxatory activities (7, 8).

Detailed knowledge of the interaction of both prospective drugs with important biomacromolecules such as DNA or serum albumins at the molecular level can be extremely important in determining their distribution in organisms and their therapeutic effect in medicine.

DNA is a molecule of great biological significance as it carries genetic information, and is a major target of drugs which in turn interfere with many intracellular processes by binding to DNA. Drug-induced modifications in the mechanical or topological properties of DNA can have a profound impact on damage and the metabolism and survival of cells (9, 10). Therefore, the investigation of drug–DNA interactions is a very important step for an understanding of the intracellular processes and plays a key role in the rational design of new potential drugs against diseases (11, 12). Indispensable to an understanding of drug–DNA interactions is the characterization of the binding modes and determination of strength of binding (13). In addition to covalent binding, several classes of non-covalent binding of drugs to DNA exist, including intercalation and groove binding (13).

Serum albumins are the most abundant plasma proteins and contribute significantly to many transport and regulatory processes. The proteins bind a wide variety of substrates such as metals, fatty acids, amino acids, hormones and an impressive spectrum of drugs (14). Serum albumins are known to contain two specialized drug-binding sites located in subdomains IIA

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and IIIA (15). The binding of hypericin to human serum albumin (HSA), or bovine serum albumin (BSA), helps to overcome difficulties in solubilization and dispersion of hypericin in aqueous physiological solution. Change in the thermal stability of albumins due to their interaction with drugs can be a good tool for investigating binding characteristics and binding constant measurements are useful for the determination of the strength of binding. Considering the importance of serum albumins in pharmacokinetics, a detailed knowledge of specific interactions of photosensitizers such as hypericin, and its derivatives emodin, alizarin, danthron and quinizarin, with serum albumins can contribute to better understanding of their role as delivery systems in organisms.

The interactions of the potential anticancer drug hypericin in photodynamic therapy with linear calf thymus DNA and transport proteins represented by BSA was the main goal of this study. The model compound emodin is a molecule which can enable better understanding of the binding effect of more complex drugs such as hypericin. The binding constants and interaction modes were determined by spectrophotometric and calorimetric methods.

Materials and Methods

Hypericin, emodin, high polymerized calf thymus DNA, human serum albumin (HSA), bovine serum albumin (BSA) were obtained by different suppliers: hypericin – KRD molecular technologies, Bratislava, Slovakia; emodin – Sigma-Aldrich, Bratislava, Slovakia; calf thymus DNA, HSA and BSA were purchased from Lambda-Life, Bratislava, Slovakia. Stock solutions of hypericin (10^{-4} mol/l, 10^{-3} mol/l, respectively) and emodin (10^{-3} mol/l) were prepared in dimethyl sulfoxide, which was acquired from Sigma-Aldrich, Bratislava, Slovakia.

Binding constants were determined by fluorescence (fluorimeter RF – 5301 PC; Shimadzu-Slovakia, Bratislava, Slovakia) and absorption spectrophotometry (absorption spectrophotometer UV-VIS 2401 PC; Shimadzu-Slovakia, Bratislava, Slovakia) using equilibrium receptor-ligand binding analysis according to Copeland (16).

Interaction of hypericin and emodin with DNA. Calf thymus DNA was dissolved in TE buffer (10 mmol/l Tris and 1 mmol/l EDTA), pH 7.4 by stirring at 4°C to obtain a concentration of 2×10^{-3} mol/l. The samples for fluorescence measurements were prepared by dissolving of stock solutions of hypericin or emodin, respectively and DNA in TE buffer. To see an influence of the DNA on drugs' emission, the concentration of DNA in relation to the drugs concentrations was changed to make the drug-DNA complexes in ratios from 1/1 to 1/600 while the amount of the drugs was kept constant. The complexes were stabilized for 14 h in the dark. However, standard theory of Copeland (16) was created for the binding of small molecules (hypericin, emodin) into macromolecules (DNA). To find an incorporation of hypericin and emodin, respectively into the DNA, we recalculated the concentration of the drugs in relation to constant DNA concentration.

The influence of drug on thermal denaturation of DNA was used to determine its mode of interaction with macromolecules. For this purpose, an absorption spectrophotometer Specord UV-VIS Analytik

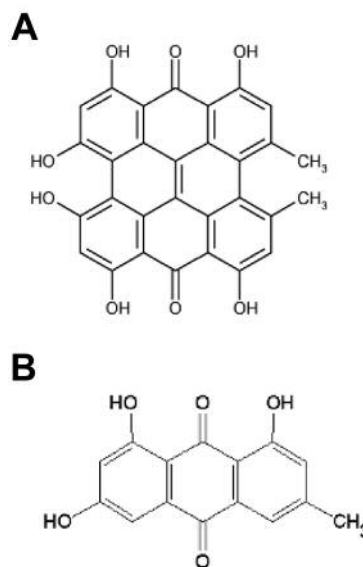


Figure 1. Chemical structure of hypericin (A) and emodin (B).

Jena (ChromSpec-Slovakia, Sala, Slovakia) connected to a Peltier module (ChromSpec) was used. This experimental arrangement enabled continuous increase of temperature at 1°C per minute. The temperature range of measurement was 20-90°C. Absorption curves of samples were recorded every 2 minutes. The samples were filtered on MillexGV (0.22 µm) (MicroChem, Bratislava, Slovakia) before measurement. Melting curves were fitted by Van't Hoff equation (17):

$$A = A_{min} + \frac{A_{max} - A_{min}}{1 + e^{\frac{\Delta H}{R} \times \left(\frac{1}{T} - \frac{1}{T_m} \right)}}$$

where A was absorbance, A_{min} the minimal measured absorbance, A_{max} the maximal measured absorbance, ΔH the enthalpy of transition, R the gas constant, T temperature, and T_m was the melting temperature.

Interaction of hypericin and emodin with BSA. Stock solutions of HSA and BSA, respectively, were prepared in phosphate buffer, pH 7.4 and used at molar concentration 10^{-4} mol/l in spectrophotometric experiments and at 2 mg/ml in calorimetric experiments.

Complexes of hypericin-BSA and emodin-BSA were prepared at three different concentration ratios: 1/1, 2/1 and 4/1. The samples were studied at the temperature range from 23-70°C using Microcal™ VP-DSC differential scanning calorimeter (DSC) (Specion, Prague, Czech Republic). The pressure was kept constant at 22.8 psi during the measurement and the heating rate was set at 60°C/h. All samples and buffers were degassed before application.

Van't Hoff analysis (18) was used for evaluation of measurements. DSC endotherms of pure BSA, the complexes of hypericin-BSA and emodin-BSA at ratios of 1/1 and 2/1 were fitted by two-state model but the DSC curves including two thermal transitions (ratios 4/1) were not fitted by two-state models. The two-

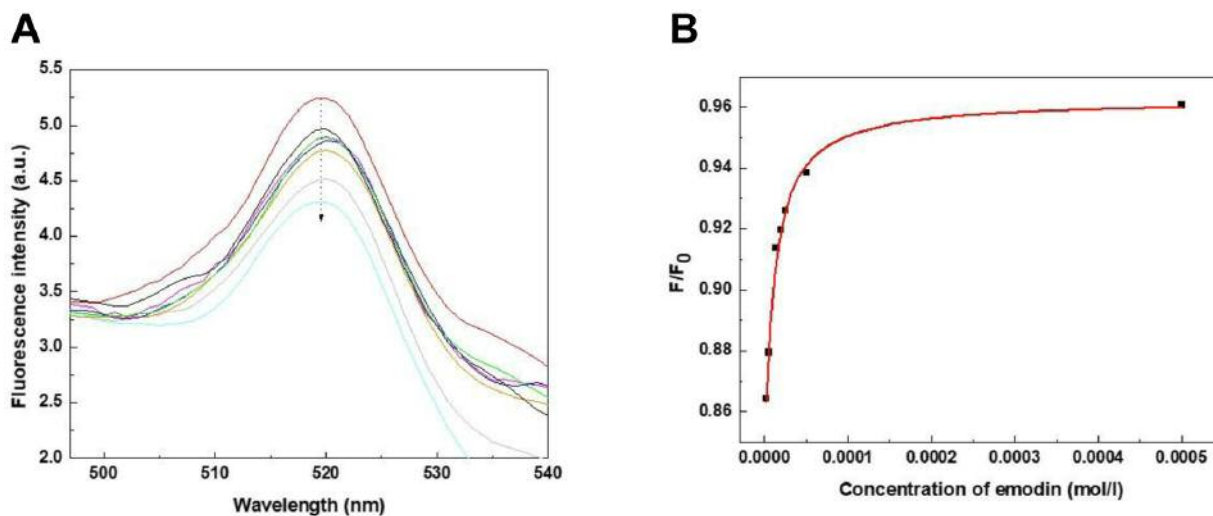


Figure 2. A: Fluorescence spectra of emodin–DNA complex. Concentration of emodin: 10^{-6} mol/l; concentration of DNA: 2×10^{-6} – 6×10^{-4} mol/l. Excitation wavelength of emodin: 440 nm. B: Binding curve of emodin–DNA formation (■) and its fit (—). Note: Increasing concentrations of emodin were recalculated in relation to constant DNA concentration. F/F_0 : F is fluorescence intensity at 520 nm of individual emodin - DNA ratios, F_0 is fluorescence intensity at 520 nm of pure DNA.

state model represents a process of biomacromolecule denaturation when the molecule is changed from native form to denaturated state. In contrast, a non-two-state model (*i.e.* more states) describes a situation when denaturation is realized through an intermediate state between the native form and totally denaturated form of the biomacromolecule.

The maximum level of dimethyl sulfoxide in the samples was less than 1% v/v in all experiments.

Results

Interaction of hypericin and emodin with linear calf thymus DNA. Absorption spectrophotometry was used to determine the mode of interaction between the drugs and DNA, and fluorescence spectrophotometry was used for measurement of binding constants. Figure 2 shows the fluorescence spectra of emodin–DNA complex including the Langmuir isotherm (Figure 2B), which gives the equilibrium dissociation constant. Semi-logarithmic graphical representation of equilibrium binding analysis (not shown) was also used in our calculations to find more exact binding constant values. Considering both graphical approaches, the binding constants (K_b) of emodin–DNA and hypericin–DNA complexes were derived as 8.1×10^4 l/mol and 4.0×10^4 l/mol, respectively. Compared with other drugs which are typical DNA intercalators, such as actinomycin D ($K_b = 1.9 \times 10^6$ l/mol) (19) and mitoxantrone ($K_b = 3.9 \times 10^5$ l/mol) (20), our obtained values do not point to intercalation as the mode of formation of these drug–DNA associations. Moreover, hypericin derivatives danthron and quinizarin interacting with DNA by electrostatic interactions (21) have similar binding constants to emodin and hypericin (22).

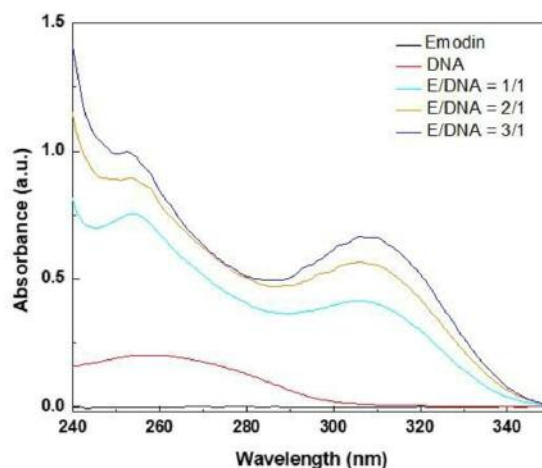


Figure 3. Absorption spectra of emodin–DNA complex. Concentration of pure emodin: 0.75×10^{-5} ; concentration of emodin in complexes: 3×10^{-5} - 9×10^{-5} mol/l; concentration of DNA: 3×10^{-5} mol/l.

Fluorescence quenching of emodin as a result of adding DNA (Figure 2A) and the value of the binding constant led us to conclude that emodin interacts with DNA by non-intercalative but groove binding mode of interaction.

The most probable binding sites in the DNA structure for hypericin molecule were found by Raman spectroscopy (23). Vibrational spectra for hypericin in complex with polynucleotides show that N7 of purines, preferentially guanine, is included in the interaction. These findings and the value of binding constant led us to supposition of groove binding mode of interaction.

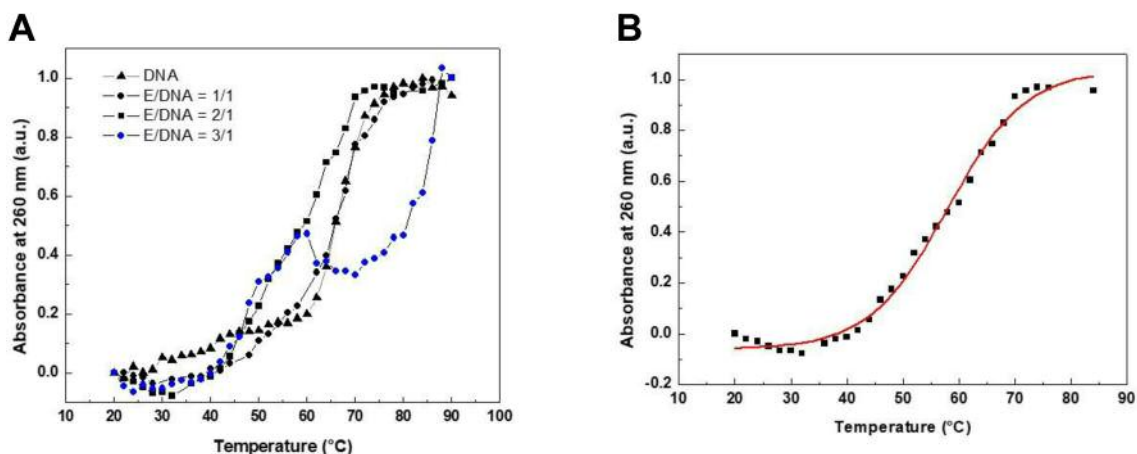


Figure 4. A: Normalized melting curves of pure DNA and emodin–DNA complexed at 1/1, 2/1 and 3/1 ratios. DNA concentration: 3×10^{-5} mol/l, emodin concentration: $3 \times 10^{-5} - 9 \times 10^{-5}$ mol/l. B: Normalized melting curve of 2/1 emodin–DNA complex (■) and its fit (—).

Table I. Thermodynamic characteristics determined from melting curves of pure DNA and emodin–DNA complexes at concentration ratios of 1/1, 2/1 and 3/1.

	1st Phase					2nd Phase	
	T_m (°C)	T_1 (°C)	T_2 (°C)	ΔT (°C)	ΔH (kJ/mol)	T_m (°C)	ΔH (kJ/mol)
DNA	67.5	56.6	75.1	18.5	338		
Emodin–DNA, 1/1	66.8	51.7	77.1	25.4	190		
Emodin–DNA, 2/1	57.0	36.9	75.5	38.6	126		
Emodin–DNA, 3/1	46.7	35.8	59.4	23.6 for AT pairs	325	94.4	121 for GC pairs

T_m : Melting temperature, T_1 : temperature at which 17% of base pairs in DNA are denatured, T_2 : temperature at which 83% of base pairs in DNA are denatured, ΔT : width of temperature interval ($T_2 - T_1$), ΔH : enthalpy of the system.

Absorption analysis (Figure 3) confirmed the results obtained by fluorescence measurements. It is known that decrease of absorbance (hypochromic effect) and small shift of absorption peak to the infrared range as a result of ligand–DNA complex formation mean an intercalation of a ligand into the DNA structure (21). The hyperchromic effect and small bathochromic shift of the DNA absorption maximum at 260 nm which can be seen in Figure 3 indicate emodin–DNA interaction *via* electrostatic forces or hydrogen bonding (24). The size of the binding constant for the emodin–DNA complex is too high to conclude that molecules interact by electrostatic forces (21, 24). The presence of emodin in solution is indicated by the emodin peak at 308 nm. Considering the above mentioned findings, we can assume interaction occurs *via* hydrogen bonds that is associated with binding to the groove of the DNA molecule.

We confirmed our above mentioned conclusions by denaturation of pure DNA and emodin–DNA complexes at

concentration ratios 1/1, 2/1, 3/1 by use of an absorption spectrophotometer connected with a Peltier module. Melting curves shown in Figure 4 show a destabilizing effect of emodin on calf thymus DNA. They were fitted using Van’t Hoff equation (17) to obtain the main thermodynamic characteristics.

From Figure 4 and Table I, a slight change in all thermodynamic characteristics was seen when emodin was mixed with DNA at different concentration ratios. The decrease in melting (denaturation) temperature points to the fact that the complex of DNA with emodin was denatured at a lower temperature than pure DNA. At the same time, the enthalpy also decreased due to complex formation. Contrary to melting temperature and enthalpy of the system, the width of the temperature interval $\Delta T = T_2 - T_1$, (T_1 is the temperature when 17% of DNA base pairs are split, T_2 is the temperature when 83% DNA base pairs are split) (see Table I) is larger, which corresponds with the above-mentioned changes in thermodynamic parameters.

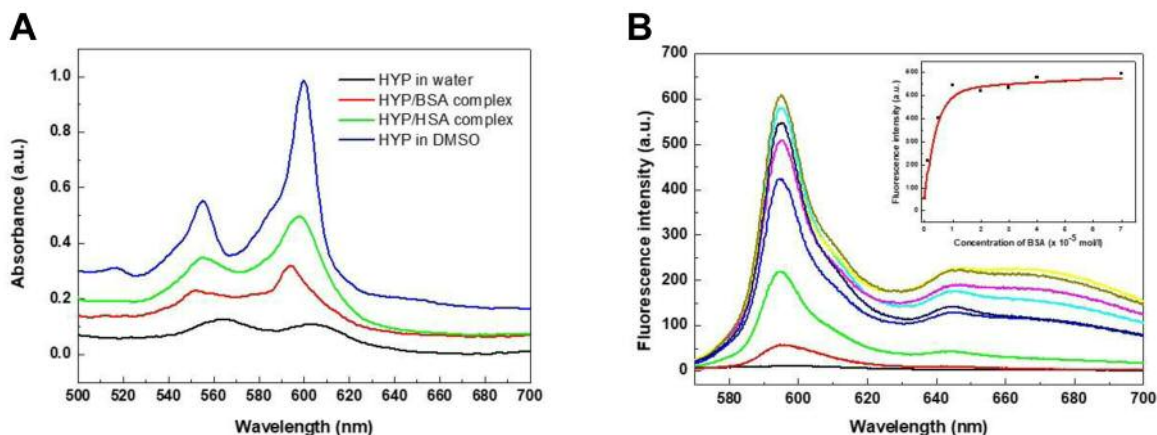


Figure 5. A: Solubilization of hypericin (2×10^{-6} mol/l) in different solvents. B: Fluorescence spectra of hypericin (10^{-7} mol/l) and hypericin–bovine serum albumin (BSA) complexes. Concentrations of BSA: (10^{-7} – 7×10^{-5} mol/l), excitation wavelength of hypericin: 550 nm. Inset: Binding curve for hypericin–BSA (■) and its fit (—).

For better presentation, Figure 4B shows experimental melting curve as well as the fitted (red) curve for the emodin–DNA complex at a concentration ratio of 2/1. Melting temperature T_m shifted from 67.5°C (pure DNA) to 57°C for the emodin–DNA complex at 2/1 concentration ratio (Table I). Furthermore, Van't Hoff enthalpy ΔH decreased from 388 kJ/mol (pure DNA) to 126 kJ/mol (emodin–DNA at 2/1), which means that less energy is needed for denaturation of 50% of base pairs of DNA in complex with emodin. The changes in all the thermodynamic parameters lead us to believe that emodin destabilizes the calf thymus DNA molecule and this destabilization is associated with groove binding interaction (25). Our findings are supported by the fact that an intercalative mode of interaction leads to thermal stabilization of DNA molecule due to drug binding (25).

It is generally supposed that small molecules like emodin bind preferentially into the minor groove of DNA, especially to regions which are rich in AT base pairs (26). In our experiments, the melting curve for emodin–DNA complex at a concentration ratio of 3/1 ratio (Figure 4A, blue curve; Table I) exhibits a two-phase character, with significant destabilization of AT regions in DNA. This finding, and the fact that the emodin molecule is half the size of the hypericin molecule, confirm our hypothesis that emodin binding into the minor groove of DNA probably occurs *via* hydrogen bonds. Shahabadi and Heidari (27) showed that the antidiabetic agent metformin, with K_b of 8.3×10^4 l/mol binds into the minor groove in DNA. Similar binding values for metformin and emodin, as well as the fact that a higher emodin concentration causes considerable destabilization of AT regions in DNA, support our conclusions.

Interaction of hypericin and emodin with serum albumins. The serum albumins (BSA and HSA) induced solubility of hypericin due to their interaction as can be seen from Figure 5. This experimental result demonstrates incorporation of the drug into a macromolecular structure.

Binding constants express the strength of interaction between macromolecules and ligands. We modified the method of Copeland (16) and regarded the protein macromolecule as the ligand. Fluorescence spectroscopy was used for measurement of changes in fluorescence of hypericin with increasing concentration of albumin (Figure 5B). Using the above modified method and fitting of Langmuir curve (Figure 5B inset), the binding constant for hypericin–BSA was determined as 2.5×10^5 l/mol. The same procedure was used for determination of the binding constant for the complex emodin–BSA $K_b = 7.5 \times 10^5$ l/mol.

The influence of both drugs on thermal stability of BSA was studied by differential scanning calorimetry and results are shown in Figure 6. It can be seen from the figure that the melting of complexes as well as the melting of pure BSA are endothermic processes. This finding leads us to hypothesize about the incorporation of the drugs within the albumin molecule during formation of their complex with albumin. An exothermic process could signify a binding of the drugs on the surface of the BSA macromolecule, such as in case of epicatechin (28) and gallotannins (29).

Miškovský *et al.* extensively studied the incorporation of hypericin into HSA and found that hypericin is rigidly held in the IIA subdomain of HSA close to Trp214 (30). Similarly, the same binding site in BSA was proposed for hypericin but the drug molecule is not incorporated so deeply in comparison with binding into HSA (30). Comparison of the

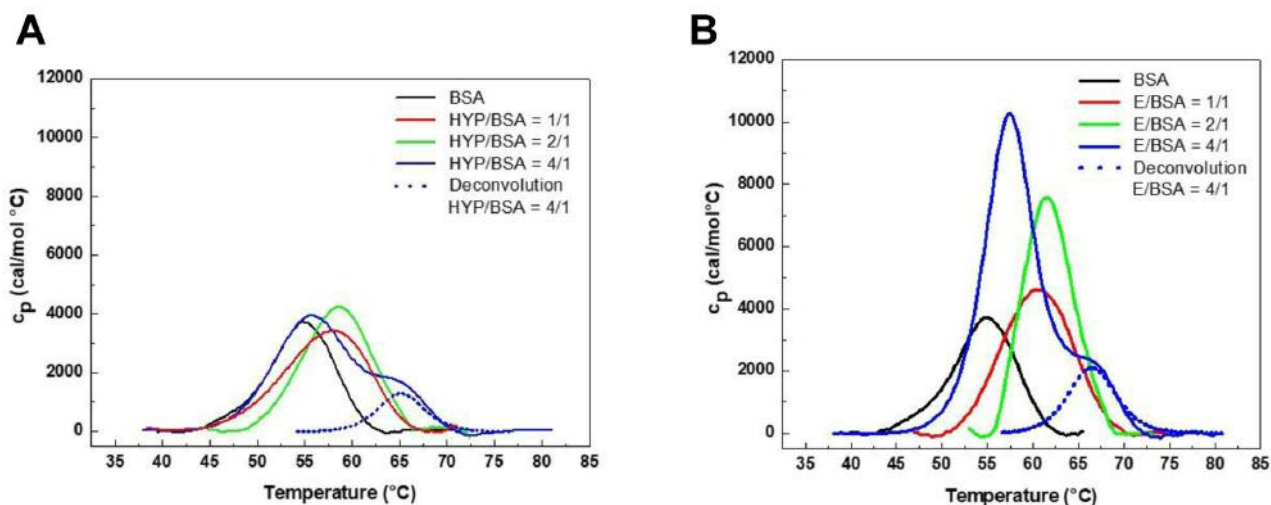


Figure 6. Differential scanning calorimetry curves of pure bovine serum albumin (BSA) and hypericin–BSA (A), and emodin–BSA (B) at concentration ratios of 1/1, 2/1 and 4/1. C_p : Thermal capacity at constant pressure.

binding constant of hypericin–HSA (7.1×10^5 l/mol) (31) with that we found for hypericin–BSA (2.5×10^5 l/mol) indirectly supports this notion.

As can be seen in Figure 6A, higher concentrations of hypericin slightly increased the stability of BSA up to 4/1 ratio. The DSC curve for the 4/1 ratio complex includes two individual endotherm peaks. Deconvolution analysis shows that the single endotherm is well approximated as the sum of three independent two-state transitions. Two transitions of the bimodal DSC curve for hypericin–BSA at 4/1 ratio are not of a two-state type. This complex molecule possibly melts as two structurally independent parts (32), one domain is destabilized while another is simultaneously stabilized (33). This hypothesis correlates with the multidomain structure of BSA. The existence of two endothermic peaks in our results may be related to a different denaturation mode of albumin domains by hypericin: the stabilization of a domain rich in fatty acids and simultaneous destabilization of poorly fatty acids saturated domain are suggested (34). The endothermic peak corresponding to the lower melting temperature ($T_m=56.79^\circ\text{C}$) (Figure 6A, Table II) is probably associated with the denaturation of albumin parts poor in fatty acids, while that at the higher melting temperature ($T_m=66.18^\circ\text{C}$) corresponds to an albumin domain which is rich in fatty acids. Finally, we propose that a higher concentration of hypericin destabilizes some parts of albumin and stabilizes other parts.

Emodin similarly to hypericin does not change the endothermic character of BSA denaturation (Figure 6B, Table II) but the stabilization of macromolecule by emodin is more significant than that by hypericin. Incorporation of emodin into HSA studied by spectrophotometric methods (35) confirmed preferential drug binding into a hydrophobic

Table II. Influence of hypericin and emodin on melting temperature of bovine serum albumin (BSA).

	Hypericin–BSA		Emodin–BSA	
	T_{m1} ($^\circ\text{C}$)	T_{m2} ($^\circ\text{C}$)	T_{m1} ($^\circ\text{C}$)	T_{m2} ($^\circ\text{C}$)
BSA	54.77		54.77	
Complex 1/1	57.45		60.78	
Complex 2/1	58.69		61.79	
Complex 4/1	56.79	66.18	58.43	67.50

cavity within HSA. With regard to structural similarities of both albumins, we assume they have the same mode of interaction. The emodin molecule stabilizes BSA due to the formation of non-covalent bonds within the emodin–BSA complex. Higher thermic parameters of emodin–BSA formation in comparison with hypericin–BSA formation (Figure 6, Table II) leads us to suppose that emodin stabilizes BSA more than hypericin does. From these findings, we can assume a deeper incorporation of emodin within BSA or the formation of stronger interaction between emodin and BSA than in the case of hypericin. Our first assumption is related to the size of emodin molecule (Figure 1), which confers greater flexibility. The K_b for the emodin–BSA complex found from our fluorescence measurements is three times higher than that for hypericin–BSA. This may explain greater stabilization of BSA by emodin in comparison with that by hypericin.

Similarly, emodin interaction with BSA at a concentration ratio of 4/1 shows the same tendency as that for hypericin with BSA.

Discussion

A better understanding of interaction of the hypericin and its derivatives with various possible cellular targets is essential for the determination of their function in biological systems.

Regarding drug incorporation into calf thymus DNA, our findings lead us to believe there that neither molecules bind into DNA by intercalation. The data summarized here show the binding constant values are not sufficient for this type of interaction. The binding constants of hypericin and emodin for interaction with DNA have values of the order of $\sim 10^4$ l/mol, while the binding constants of typical intercalators such as ethidium, daunomycin and psoralen reach value of more than 10^6 l/mol (36).

The influence of emodin on denaturation of DNA indicates the binding into a groove and incorporation of a drug takes probably place in the minor groove *via* hydrogen bonds. Thermal destabilization of the DNA molecule as a result of association with the drug clearly demonstrates the groove binding mode. The binding of the drugs on the surface of the DNA molecule *via* slight electrostatic forces is also very improbable since the complex should be stabilized if this state is reached. Moreover, the binding constant values are too high for this type of interaction.

Comparing the structure of hypericin and emodin molecules considering their binding characteristics during complex formation with DNA, we hypothesize that hypericin binds into the major DNA groove, this is supported by results obtained by Kočíšová *et al.* (23).

The fact that serum albumins are good solvents for both drugs has led us to a claim about their incorporation within macromolecules. The binding of hypericin into HSA and BSA was described in detail by Miškovský *et al.* (30) and their findings of preferential binding hypericin into the hydrophobic cavity in IIA subunit of albumins are clearly accepted. Here, we have confirmed the deeper incorporation of hypericin into HSA in comparison with its incorporation into BSA proposed by Miškovský *et al.* (30).

In conclusion, we believe that potential anticancer agent hypericin and its model compound emodin interact with important biomacromolecules such as DNA and serum albumins. We determined strength of interaction expressed by binding constants that were found to be 4.0×10^4 l/mol for hypericin–DNA and 8.1×10^4 l/mol for emodin–DNA complex. The binding constants point to medium strength binding between drugs and DNA. We suppose an interaction of drug with the DNA groove, mediated by hydrogen bonds. Both molecules stabilize BSA and probably bind into the hydrophobic cavity in the IIA subunit but their localization within the molecule is different. The size of the emodin molecule, which is half that of the hypericin molecule, indicates its higher flexibility and possibility of deeper incorporation into the BSA hydrophobic cavity.

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