BINDING OF ANTITUMOR COMPOUNDS TO WHEAT PROTEIN

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ABTSRACT

Binding of porphyrins to biological macromolecules is important to be studied as an approach to keeping them in stable, effective and safe dosage forms. In this work we show the interaction of three porphyrin compounds: Hematoporphyrin IX, Mn- and Fe-porphyrins with plant lectin Wheat germ agglutinin (WGA). Porphyrin binding to WGA was monitored by the changes in the intrinsic protein fluorescence. Conformational changes due to the formation of WGA-porphyrin complexes, were shown by the hyperbolic binding curves. The calculated dissociation constants K_D (0.16 μM to 0.24 μM) indicate high affinity of WGA for the three porphyrins. Most probably they occupy the same high affinity sites. In conclusion, since WGA binds cancer cells and interacts with antitumor compounds, it could be useful as a drug delivery molecule.

Biotechnol. & Biotechnol. Eq. 2013, **27**(3), 3857-3860

Keywords: porphyrins, wheat germ agglutinin, protein

Introduction

The ideal purpose of the efficacious cancer therapy is to destroy cancer cells without affecting the healthy ones. A new class of therapeutically important compounds widely applied in the therapy of cancer are porphyrins. One of the best studied and well characterized porphyrin compounds approved for clinical application in photodynamic therapy (PDT) of cancer is Hematoporphyrin IX (7). Recent studies have shown that metal-based drugs are attractive for treatment of cancer, as well as diabetes, inflammatory and cardiovascular diseases, psoriasis, etc. Besides this, complexes of iron have shown remarkable antiproliferative properties (27). Also, ferrocifenes exhibit anticancer activities against hormone dependent and hormone independent breast cancers (24, 29).

However, similar to other anticancer agents, porphyrins accumulate not only in tumor tissues, but cause damage to normal ones as well. Therefore, to enhance the selective binding to cancer cells by porphyrins, some plant lectins were used to deliver the sensitizers (19).

Lectins are carbohydrate-binding proteins that play an important role in biosignaling, cell–cell and cell–matrix interactions etc., for which they are appropriate molecules for the targeting of anticancer drugs. Interestingly, some lectins such as: snake gourd *Trichosantes anguina* seed lectin, Concanavalin A, peanut lectin and jacalin bind photosensitizers (4, 9, 10, 11, 13, 17, 18).

We chose to study the plant lectin Wheat germ agglutinin (WGA) isolated from *Triticum vulgaris*, since this lectin binds more strongly tumor cells than normal ones (1, 2, 3). This protein binds and enters prostate cancer cells (Du-145) by recognizing the accessible carbohydrates on the cell membrane such as: sialic acid and N-acetyl-D-glucosamine (8).

WGA is a 34 kDa protein composed of two subunits, each containing four domains arranged in tandem. It is an appropriate delivery molecule for transportation of anticancer agents, such as doxorubicin, paclitaxel, and bufalin, to cancer cells (12, 20, 30, 31).

In this regard, we investigated the binding of WGA to three anticancer agents: Hematoporphyrin IX, Mn- and Fe-porphyrin.

Our results show the formation of WGA-porphyrin complexes that probably could have further application on tumor cells.

Materials and Methods

Materials

5,10,15,20-Tetrakis(N-methyl-4-pyridyl)porphyrin-Mn(III)pentachloride (MnTMPyP), 5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrin-Fe(III)chloride (FeTPPS) and 8,13-Bis(1-hydroxyethyl)-3,7,12,17-tetramethylporphyrin-2,18-dipropionoc acid - Hematoporphyrin IX (HP IX) were purchased from Porphyrin Systems (Lüebeck, Germany). Lectin from *Triticum vulgaris* (wheat) was purchased from Sigma. The protein was dissolved in PBS (20 mM phosphate buffer containing 0.15 M NaCl, pH 6.8) and the concentration of WGA was determined spectrophotometrically, using the extinction coefficient (ε_{280}) calculated from the aromatic amino acid residues. The concentrations of MnTMPyP and HP IX were calculated spectrophotometrically by their molar extinction coefficients: $\varepsilon_{462} = 0.95\times10^5\ M^{-1}\cdot cm^{-1}$ for MnTMPyP and $\varepsilon_{394} = 1.55\times10^5\ M^{-1}\cdot cm^{-1}$ for HP IX. FeTPPS concentration was calculated by its molecular weight.

Fluorescent measurements

Steady-state fluorescent measurements were performed with a Shimadzu fluorometer (Japan). To avoid detection of the Tyr emission, the protein sample was excited at 295 nm with an excitation band pass of 5 nm and an emission band pass of 10 nm. Total fluorescence was calculated after normalization of the fluorescence spectra and correction for dilution. In order to account for the inner filter and the self absorption effects, the experiments were always carried out on samples with absorbance less than 0.05 OD. All measurements were made at 25 °C with the temperature of the samples determined in the cuvette with an accuracy of ± 0.2 °C.

Binding of porphyrin compounds to WGA

The porphyrin–protein interactions were studied by the fluorescence spectroscopy method, which is considered most suitable due to its intrinsic sensitivity and simplicity. For this purpose, WGA (1.5 μM) was incubated at 4 ºC overnight, with increasing concentrations of HP IX and MnTMPyP. Also, WGA (1.5 μM) was titrated with increasing concentrations of FeTPPS. Low porphyrin concentrations were used (0.07 μM to 0.8 μM) in order to minimize artifacts due to aggregation phenomena. Experimental data were processed by a non-linear regression analysis computed with the Graph Pad Prizm program. The good fit of the models was confirmed by SPSS and the method described by Kolikov et al. (15, 16) was used as well. The formation of WGA-porphyrin complexes (0.16 μM to 0.24 μM) was investigated by monitoring the changes in the intrinsic protein fluorescence emission at 350 nm. The maximal decrease of the protein fluorescence due to the saturation of the binding sites by the porphyrin ligand (Fmax) was calculated from the incubation and titration data.

Competitive experiments

Competitive experiments were performed in order to compare the affinity of the three porphyrins to accommodate the same type of binding sites. For this purpose three experimental sets were done: 1) titration of WGA–MnTMPyP complex with HP IX and WGA–HP IX complex with MnTMPyP, 2) titration of WGA–FeTPPS complex with HP IX and of WGA–HP IX complex with FeTPPS, and 3) titration of WGA–MnTMPyP complex with FeTPPS and of WGA–FeTPPS complex with MnTMPyP.

Results and Discussion

It has been shown that WGA interacts with carbohydrates (21), hormones (6) and hydrophobic compounds as: ANS and TNS (25). Of major interest to us is to study the interaction of WGA with three photosensitizers: hematoporphyrin IX, Mn- and Fe-porphyrin.

First, we tested the binding of WGA to HP IX, which is a component of *Photofrin* and is applied in pre-clinical and clinical PDT. This photosensitizer is approved for the treatment of bladder, esophageal, lung cancers (7). Therefore, its complex with WGA could be important for the new anticancer targeted treatments.

We studied the formation of WGA–porphyrin complexes by the changes in the intrinsic protein fluorescence. We found that the binding of HP IX to WGA caused a significant decrease in the Trp fluorescence intensity, which is indicative of conformational changes within the protein molecule. Similarly, fluorescence spectroscopic studies have demonstrated that the typical spectrum of WGA changes after interaction with $(GlcNAc)_{2,3,4}$ oligosaccharides (21). The experimental data have shown that WGA interacted with HP IX with high affinity (K_D of 0.24 μM ± 0.05 μM), similar to the binding of HP IX to myoglobin, studied by spectroscopic methods, according to which HP IX remained in a monomer form at low concentrations (0.4 μM to 1 μM) (26). **Fig. 1** shows the hyperbolic binding curve, which indicates the presence of one type of binding sites. The residuals graph (shown in the insert of **Fig. 1**) shows the normal distribution of the residuals, which proves that the obtained equation of the non-linear regression is a good fit of the data.

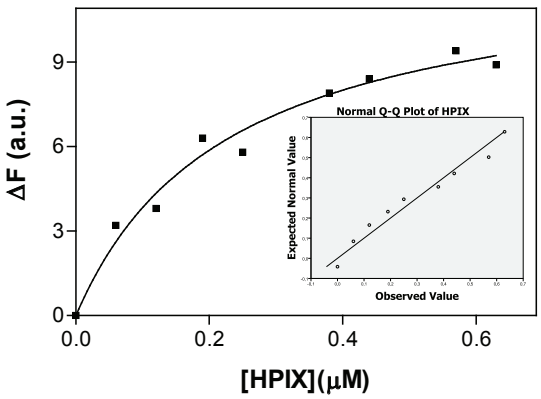

Fig. 1. Formation of WGA–HP IX complex, studied by means of intrinsic protein fluorescence. Excitation was set at 295 nm. Insert: residual plot ($R^2 = 0.98$).

We also investigated the binding of WGA with MnTMPyP by the changes in the Trp fluorescence after incubation with increasing concentrations of this photosensitizer. For the formation of the WGA–MnTMPyP complex we measured a dissociation constant K_D of (0.23 ± 0.05) μM. The emission maximum position was at 350 nm, characterizing the exposition of the Trp amino acids located on the protein surface. The hyperbolic model of the binding suggests one type of high affinity site for MnTMPyP (**Fig. 2**). Recently, it has been found that MnTMPyP shows synergistic cytostatic effect with ascorbic acid upon different cancer cell lines such as prostatic, pancreatic and hepatic cancer cells. The cytostatic effect was a result of combined treatment that mediates an increase of the cellular levels of H_2O_2 and $HO^{\bullet}$ in cancer cells (28).

It has been shown that Fe compounds demonstrate interesting therapeutic potential (27, 29). Recent studies have revealed that FeTPPS is efficacious in ROS reactions and induces cell death in G361 (a human melanoma cell line) (14). This urged us to study WGA–Fe porphyrin interactions. In **Fig. 3** the decrease of fluorescence spectra is shown, registered

by protein fluorescence. Our study showed one type of high affinity binding sites for FeTPPS within the WGA molecule. From the representative titration curve shown in **Fig. 3** (insert), we calculated an affinity constant K_D of (0.16±0.03) μM, which is one order higher than the affinity of human prion protein to Fe-porphyrin (22).

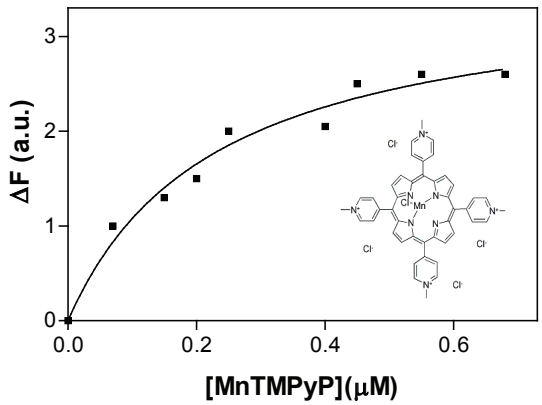

Fig. 2. Binding of MnTMPyP to WGA (λ_{exc} at 295 nm). Insert: structure of Mn-porphyrin.

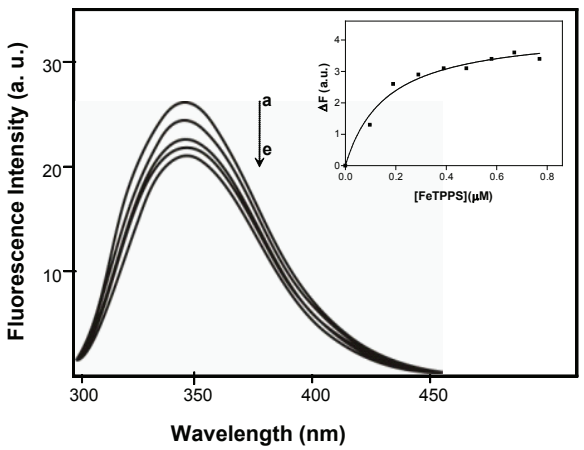

Fig. 3. Variation of the fluorescence emission spectra of WGA (1.5 μM) in the presence of FeTPPS without porphyrin (**a**); and with 0.1 μM to 0.8 μM porphyrin (**b–e**). Insert: hyperbolic binding curve showing the interaction of WGA with FeTPPS.

Our study reports spectroscopic data about the affinity of WGA to HPIX, MnTMPyP and FeTPPS. This affinity is similar to the recently reported affinity showing the interaction of WGA with two water-soluble cationic porphyrins: (meso-tetra(4-N-ethylacetatepyridyl)porphrin (H2NEAE-pp) and the Zn(II) complex (ZnNEAE-pp), also studied by an optical biosensor approach (23). Similar affinity has been shown by this protein toward FeTMPyP and PdTPPS (5). Our experimental results describe the binding activity of WGA to three porphyrins. Wright et al. (32) reported about two types of sites within the protein structure: high and low affinity sites. We estimated similar changes of the intrinsic Trp fluorescence, caused by the binding of HP IX, MnTMPyP and FeTPPS, which suggests that they could probably accommodate the same type of high-affinity binding site. This hypothesis was confirmed in three separate sets of competitive experiments (described in Materials and Methods) analyzing the consequent interaction of the two porphyrins which showed that there are no changes in the Trp fluorescence upon the porphyrin binding.

Conclusions

In summary, the results of this investigation suggest that, since WGA has the ability to bind cancer cells and interacts with porphyrins, it could be useful as a drug delivery molecule.

Acknowledgements

The authors are thankful for the support from the Bulgarian National Science Fund with the Ministry of Education and Science under contracts DTC No. 02/35 and DRG 02-4/2010.

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