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Attenuation of acridine mutagen ICR-191 — DNA interactions and DNA damage by the mutagen interceptor chlorophyllin

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ABSTRACT

We have investigated the ability of chlorophyllin (CHL) to interact with acridine mutagen ICR-191 (2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine) and also its ability to decrease binding of ICR-191 to DNA in a simple three-component competition system: CHL-ICR–DNA. Our data indicate a strong association of ICR-191 with CHL, stronger even than the association of ICR-191 with DNA. Calculations based on the measured affinity data show that a two- to three-fold excess of CHL reduces by about two-fold the concentration of the mutagen-DNA complex. We also exposed human leukemic HL-60 cells to ICR-191 in the absence and presence of CHL and measured the mutagen-induced DNA damage. The extent of DNA damage was assessed by analysis of histone H2AX phosphorylation. While ICR-191 induced significant increase in expression of phosphorylated H2AX (γH2AX), particularly in DNA replicating cells, this increase was totally abolished in the cells treated with ICR-191 in the presence of CHL.

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1. Introduction

Chlorophyllin (CHL) is a water-soluble derivative of chlorophyll differing from the latter by having copper instead of magnesium as a cofactor. Because of anti-mutagenic and anti-oxidant properties both chlorophyll and CHL are in center of interest of many research laboratories. Several studies reported that CHL protects cells against mutagenic effects of benzo[a]pyrene [1–4], cyclophosphamide [3], heterocyclic amines [5–7], aflatoxin [8–12], heavy metals [13], and ionizing radiation [14]. Because of diversity of the mutagens neutralized by CHL and different modes of their genotoxicity it is quite apparent that more than a single mechanism may be responsible for the CHL-protective effects. One mechanism may involve the direct anti-oxidant properties of CHL. Indeed, the ability of CHL to scavenge reactive oxygen species (ROS) *in vitro* and *in vivo* was documented [15,16].

Another mechanism by which CHL neutralizes mutagens stems from its ability to form complexes with the aromatic mutagens [17–20]. The complexes are thought to be maintained *via* stacking (π–π) interactions between the flat aromatic molecules of the mutagen and the porphyrin rings of CHL. Because the mutagen is sequestered within the complex its concentration in monomeric, i.e. active form in solution, is reduced. Thus, by capturing mutagen molecules CHL neutralizes them preventing their uptake by the cells and subsequent interaction with DNA. It was proposed to define the agents with such properties as the *interceptor molecules* [21–24]. Similar to chlorophyllin, caffeine and pentoxifillin were shown to be interceptors of several aromatic compounds such as acridine orange, quinacrine mustard and acridine mutagens ICR-191 and ICR-170, attenuating their mutagenic and cytotoxic effects and preventing interaction with DNA [25–29].

In prior studies we explored molecular interactions that may be responsible for the interceptor properties of CHL *vis-à-vis* several intercalators [22,23]. Towards this end using optical spectroscopy we have studied interactions between the intercalators and DNA in the presence and absence of CHL. We have shown for example, that CHL forms complexes with acridine orange, quinacrine mustard and the antitumor drug doxorubicin, with association constants (K_a's) 7.0×10^5, 3.2×10^5 and 3.3×10^5 M^{-1}, respectively [22]. Applying experimental analysis to the three-component interactive system: intercalator–DNA–CHL we observed that CHL attenuated binding of these intercalators to DNA thereby showing the interceptor activity [23]. In the present study using similar experimental approach we investigated interactions between the acridine mutagen ICR-191

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and DNA in the absence and presence of CHL. ICR-191 is an aromatic mutagen having a three-ring structure, it intercalates into DNA and has also mustard-like alkylating properties [30,31]. We expected, therefore, that similar as in the case of intercalators studied by us before [23] CHL will also have the interceptor activity towards ICR-191.

Parallel to the biophysical studies we presently also assessed the potential of CHL to neutralize genotoxic activity of ICR-191. Towards this end we exposed human promyelocytic HL-60 cells to ICR-191 in the absence and presence of CHL and the extent of genotoxic effect of ICR-191 was measured by flow cytometry by means of immunocytochemical detection of phosphorylation of histone H2AX. Phosphorylation of histone H2AX on *Ser*-139 (phosphorylated H2AX has been named γH2AX) is considered to be a marker of DNA damage, particularly the induction of DNA double-strand breaks (DSBs) [32,33]. Cytometric detection of γH2AX was shown to provide a convenient tool to measure extent of DNA damage induced by variety of genotoxic agents including radiation [34,35], antitumor drugs [36,37], exogenous and endogenous oxidants [38] and environmental carcinogens [39].

2. Materials and methods

2.1. Spectral studies

2.1.1. Reagents

Chlorophyllin (sodium-copper salt), Acridine Mutagen ICR-191 (6-Chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride), (both from Sigma Chemical Co, St. Louis, MO, USA), Tris (Tris-(hydroksymethyl)-aminomethane) (Fluka Chemie AG, Buchs, Switzerland). All solutions were prepared in Tris–HCl (30 mM), NaCl (50 mM) at pH 7.4.

2.1.2. Estimation of ICR–CHL association constant

Absorption and fluorescence emission measurement of ICR-191, CHL and mixtures of these compounds were done using Cary 300 spectrophotometer (Varian, Australia) and LS 50B spectrofluorimeter (Perkin Elmer, UK), respectively. Titrations were carried out at constant ICR-191 concentrations (8, 10 or 12 μM) while altering CHL concentration within the range between 0.9 to 60.0 μM. Absorption was measured in 3.0 cm width cuvettes while fluorescence emission was measured using 0.4×1.0 cm cuvettes with 5 nm excitation-, and 8 nm spectral width emission-slits, at λ_{ex}=422 nm. The temperature of the cell-housing block was kept at 295±1 K. The data were processed using Prism 4 (GraphPad Software Inc, San Diego, CA, USA). Spectrophotometric and spectrofluorimetric titrations allowed us to estimate association constant K_a and molar absorption coefficient of the complex, assumed to have the components in 1:1 proportion, according to the method applied before [22].

Fluorescence spectrum was corrected with respect to the reabsorption and the inner filter effect as described before [40].

To estimate K_a from the spectrofluorimetric measurements we applied nonlinear regression for fluorescence intensity at λ=492 nm according to (Eq. (1)).

$$\Delta F = 0,5\frac{C_A^0 + C_B^0 + \frac{1}{K} - \sqrt{\left(C_A^0 + C_B^0 + \frac{1}{K}\right)^2 - 4C_A^0 C_B^0}}{C_A^0}(F_{AB} - F_0) \quad (1)$$

where:

C_A^0 total ICR-191 concentration
C_B^0 total CHL concentration
F_{AB} fluorescence of ICR-191 totally bound to CHL
F_0 fluorescence of ICR-191 in C_A^0.

2.1.3. Estimation of ICR–DNA association constant

Absorption of ICR-191 and DNA separately as well as in mixture have been measured at constant ICR concentration within a range between 8.0 and 10.1 μM while DNA concentration was variable ranging between 1 to 60 μMbp. Absorption measurements were carried out in cuvettes of l=3 cm width while 0.4×1.0 cm width cuvettes were used for fluorescence measurement. The association constant K_i and binding site size in DNA (n) were estimated according to the McGhee-von Hippel model [41]. The K_i association constant and molar extinction coefficient of the DNA-bound intercalator ICR-191 were estimated by the least squares regression analysis as described [23]. Spectral fragments within 360–500 nm have been used in which ICR-191 has absorption band while DNA is not absorbing light. A correction for light scattering by DNA molecules was applied as described before [23].

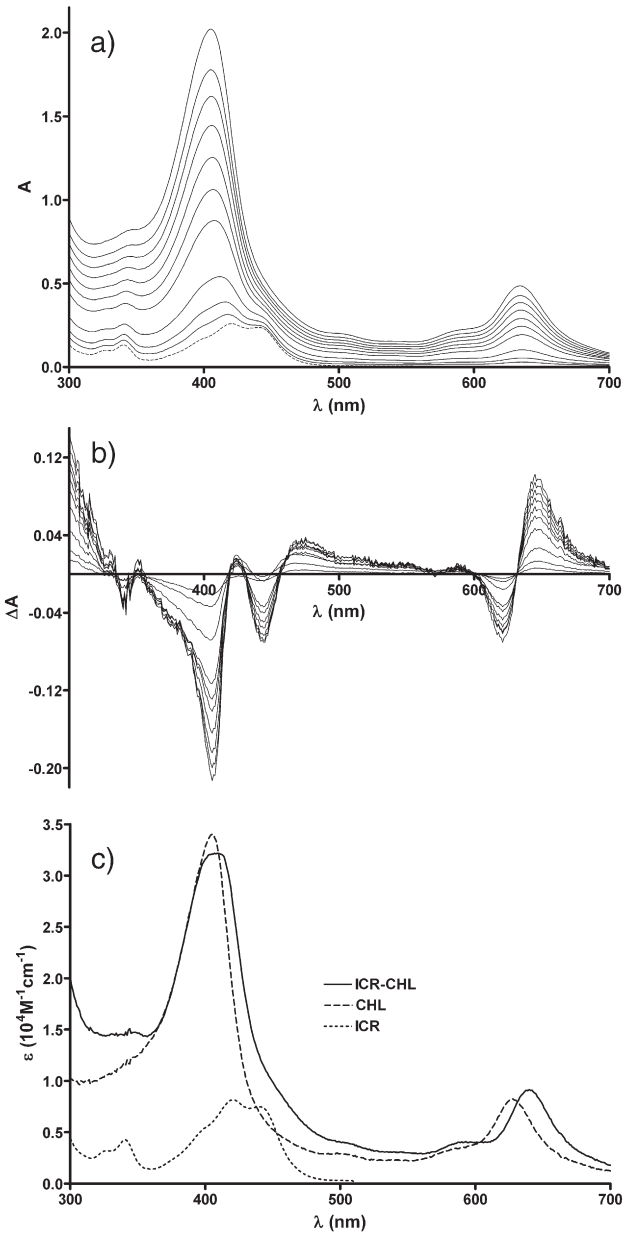

Fig. 1. a) Absorption spectra of ICR-191 (broken line) and mixtures of ICR and CHL (solid lines), ICR was at 8 μM, while CHL concentration varied from 1 to 20 μM, from bottom to top; l=3 cm; b) respective differential spectra; c) absorption spectra of ICR (dotted line), CHL (dashed line), and computed spectrum of ICR–CHL complex (solid line).

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