Interceptor effect of C_{60} fullerene on the in vitro action of aromatic drug molecules

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Abstract C_{60} fullerenes are spherical molecules composed purely of carbon atoms. They inspire a particularly strong scientific interest because of their specific physicochemical properties and potential medical and nanotechnological applications. In this work we are focusing on studying the influence of the pristine C_{60} fullerene on biological activity of some aromatic drug molecules in human buccal epithelial cells. Assessment of the heterochromatin structure in the cell nucleus as well as the barrier function of the cell membrane was performed. The methods of cell microelectrophoresis and atomic force microscopy were also applied. A concentration-dependent restoration of the functional activity of the cellular nucleus after exposure to DNA-binding drugs (doxorubicin, proflavine and ethidium bromide) has been observed in human buccal epithelial cells upon addition of C_{60} fullerene at a concentration of ~10^{-5} M. The results were shown to follow the framework of interceptor/protector action theory, assuming that noncovalent complexation between C_{60} fullerene and the drugs (i.e., hetero-association) is the major process responsible for the observed biological effects. An independent confirmation of this hypothesis was obtained via investigation of the cellular response of buccal epithelium to the coadministration of the aromatic drugs and caffeine, and it is based on the well-established role of hetero-association in drug-caffeine systems. The results indicate that C_{60} fullerene may reverse the effects caused by the aromatic drugs, thereby pointing out the potential possibility of the use of aromatic drugs in combination with C_{60} fullerene for regulation of their medico-biological action.

Keywords DNA-binding drugs · C_{60} fullerene · Caffeine · Human buccal epithelial cells · Hetero-association

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Introduction

In recent years, there has been a rapid growth of interest in the physico-chemical properties of pristine C_{60} fullerenes due to their extensive application in medicine and nanobiotechnology (Anilkumar et al. 2011; Cataldo and Da Ros 2008). It has been shown previously that C_{60} fullerene is able to penetrate through the cell membrane (Foley et al. 2002; Prylutska et al. 2012; Qiao et al. 2007) and exhibits antioxidant properties (Prylutska et al. 2008). Being non-toxic, at least at low concentrations (Andrievsky et al. 2005; Aschberger et al. 2010; Kolosnjaj et al. 2007; Prylutska et al. 2007a), pristine C_{60} fullerenes are able to suppress the growth of malignant tumors (Prylutska et al. 2011a, b).

Recently, it was found that the in vivo application of pristine C_{60} fullerene together with the DNA-binding anticancer drug doxorubicin results in enhancement of the biomedical effect of the latter (Prylutska et al. 2011c). This result creates a scientific basis for the new technology of C_{60} fullerene application in anticancer therapy based on simultaneous administration of an anticancer drug and a C_{60} fullerene. Taking into account that C_{60} fullerene has a saturated π-electron system and therefore can form complexes with aromatic DNA intercalators, including antineoplastic antibiotics and mutagens (Buchelnikov et al. 2013; Evstigneev et al. 2013; Mchedlov-Petrossyan et al. 2001), it was suggested that a non-covalent complexation in the 'drug-fullerene' system (also referred to as hetero-association) is a key mechanism causing the observed biological synergism (Evstigneev et al. 2013). It has been known for quite a long time and so far been observed in proliferating cells or bacterial systems [see for review (Evstigneev et al. 2008; Evstigneev 2010, 2013)] that hetero-association regulates the action of the biologically active compounds during simultaneous administration of combinations of different aromatic molecules, one of which is the main drug of interest, and the other one is an interceptor molecule. This phenomenon is referred to as an 'interceptor' mechanism. However, the study of such a mechanism is complicated by possible influence from other molecular processes, one of which is most often referred to as a 'protector' mechanism, i.e., the drug/interceptor competition for the binding sites on DNA (Buchelnikov et al. 2012; Evstigneev 2010, 2013; Evstigneev et al. 2011; Goluński et al. 2013). Investigation of the interceptor and protector mechanisms for DNA-binding drugs used in combination is the subject of the theory of interceptor/protector action (Buchelnikov et al. 2012; Evstigneev et al. 2008, 2011; Evstigneev 2010). Unfortunately, the applicability of the protector mechanism within the system containing C_{60} fullerene cannot be estimated on the basis of the currently available literature data. This is because the characteristics of the C_{60} fullerene binding with DNA under conditions close to the physiological ones are unknown except for the data reported in the theoretical work (Zhao et al. 2005), which has not yet received experimental confirmation.

In the present work, human buccal epithelial cells were used as a biological test system in order to investigate the possible role of the interceptor mechanism in regulation of the biological activity of DNA-binding aromatic compounds (referred to as BACs for 'biologically active compounds') in the presence of C_{60} fullerene. The relatively large size of these cells allows one to observe the structural changes in nuclear chromatin and the state of the cellular membranes using a simple biological microscope and with minimal intervention into the system while maintaining good reproducibility of the results (Shckorbatov et al. 2009a, b). In addition, buccal epithelial cells are non-proliferating and, to the best of our knowledge, the studies of the combined effects of aromatic drugs have been reported only for proliferating cell systems so far (see above). Assuming that C_{60} fullerene acts via the interceptor mechanism, the observed effects should not depend on the type of cellular system used. Hence, the general features of its manifestations should be similar in proliferating and non-proliferating cell cultures, and buccal epithelial cells may serve as a good test system to verify the hypothesis about the role of the interceptor mechanism in the regulation of biological activity of DNA-binding BACs.

A range of typical DNA-binding aromatic BACs has been used in the present study: the antitumor antibiotic doxorubicin (DOX), aromatic mutagen proflavine (PF) and ethidium bromide (EB). These drugs form stable non-covalent complexes with C_{60} fullerene (Buchelnikov et al. 2013; Evstigneev et al. 2013) and were previously characterized as the ligands that follow the mechanism of interceptor/protector action when used in combination with various types of aromatic interceptor molecules (Buchelnikov et al. 2012; Evstigneev et al. 2006, 2008; Evstigneev 2010, 2013; Larsen et al. 1996; Piosik et al. 2010; Traganos et al. 1991).

Materials and methods

Epithelial cells

Human buccal epithelial cells were collected from three adult donors with a sterile obtuse spatula and washed three times by centrifugation (2 min at 3,000 rpm) in a phosphate buffer of the following composition: 3.03 mM phosphate buffer (pH 7.0) with addition of 2.89 mM calcium chloride. Cells were then diluted with the same buffer solution (further referred to simply as 'buffer solution') to a concentration of 1–2 $\times$ 10^5 cells per ml, as determined by microscopic count, and 0.5-ml aliquots were pipetted into the Eppendorf tubes. During the course of the experiment, no visible changes in

the structure of the nucleus and cell membrane, or in the cell nuclei electronegativity and chromatin state in the investigated cells were observed in the control group.

Compounds

C_{60} fullerene: The pristine C_{60} fullerene aqueous colloidal solution (C_{60}FAS) used for the experiments was prepared as follows (Prylutska et al. 2007b; Scharff et al. 2004): a saturated solution of C_{60} fullerene (purity 99.5 %) in toluene was mixed with the same amount of distilled water, and the resulting two-phase system was ultra-sonicated until the toluene had completely evaporated. Following this, the yellow-colored water phase was filtered to remove undissolved C_{60} fullerene. A C_{60}FAS sample with a maximum concentration of C_{60} fullerene in water of 0.1 mg ml^{-1} (1.39×10^{-4} M) was prepared.

The state of C_{60} fullerene was monitored using atomic force microscopy (AFM; "Solver Pro M" system; NT-MDT, Russia). The sample was deposited onto a cleaved mica substrate (V-1 grade, SPI supplies) by precipitation from an aqueous solution droplet. Sample visualization was carried out in semi-contact (tapping) mode after complete evaporation of the solvent. NSG10 (NT-MDT) probes were used. The AFM picture (Fig. 1) clearly demonstrates the presence of single C_{60} molecules in water, as well as their clusters with a typical 2–100-nm range of diameters, which is in agreement with theoretical calculations (Bulavin et al. 2000; Prylutskyy et al. 1999, 2001), a small-angle neutron (Scharff et al. 2004) and dynamic light scattering (Prylutskyy et al. 2013) data.

Doxorubicin (DOX)

A stock solution of DOX (Doxoribicin Teva, The Netherlands) with a concentration of 2 mg ml^{-1} (3.448×10^{-3} M) was prepared by dissolving the compound (10 mg) in 5 ml of buffer solution. The initial stock solution (0.058 ml) was used to obtain a final working solution (2×10^{-4} M) by addition of 0.942 ml of buffer solution.

Ethidium bromide (EB)

A stock solution of EB (Sigma, USA) with a concentration of 1×10^{-3} M was prepared by dissolving the compound (1.97 mg) in 5 ml of buffer solution. The initial stock solution (0.4 ml) was used to obtain the final working solution (4×10^{-4} M) by addition of 0.6 ml of buffer solution.

Proflavine (PF)

A stock solution of PF (Sigma, USA) with a concentration of 1×10^{-3} M was prepared by dissolving the compound (1.05 mg) in 5 ml of buffer solution. The initial stock solution (0.4 ml) was used to obtain the final working solution (4×10^{-4} M) by addition of 0.6 ml of buffer solution.

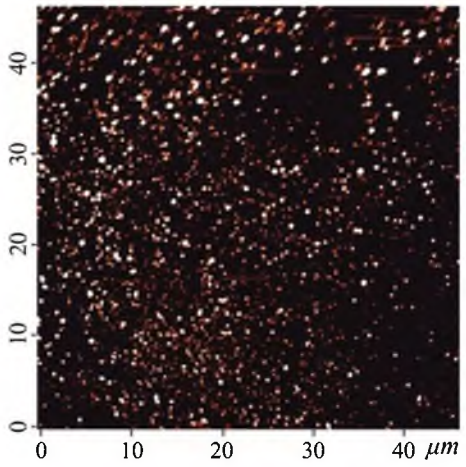

Fig. 1 The AFM image of the distribution of C_{60} fullerenes in water at a concentration of 0.1 mg ml^{-1}

Caffeine (CAF)

A stock solution of CAF (Sigma, USA) with a concentration of 1×10^{-2} M was prepared by dissolving the compound (9.71 mg) in 5 ml of buffer solution.

Chromatin state evaluation

The change in functional state of the cell nucleus is correlated with the structural transitions from heterochromatin to euchromatin. An increase in the quantity of heterochromatin granules (HGQ) indicates a decrease in the transcriptional activity of the nucleus. The evaluation of the HGQ parameter was performed using the method described in detail in (Shckorbatov et al. 2009b). Exposed cells and the control sample were stained with 2 % solution of indigo carmine in 45 % acetic acid. Cell nuclei were visually examined under the MICROmed XS-3330 microscope using 1,000× magnification (Fig. 2a). In each sample, the HGQ parameter was determined for 30 randomly selected cells. It was shown previously (Shckorbatov et al. 2009b) that this number is optimal, i.e., a further increase in the number of analyzed cells does not result in a significant decrease of standard error; however, it remarkably slows down the whole analysis. The person investigating the HGQ was 'blind' to the exposure conditions, and all measurements were made by the same person.

Membrane state evaluation

The state of the cell membranes was assessed by measuring the percentage of cells stained in vitro by indigo carmine

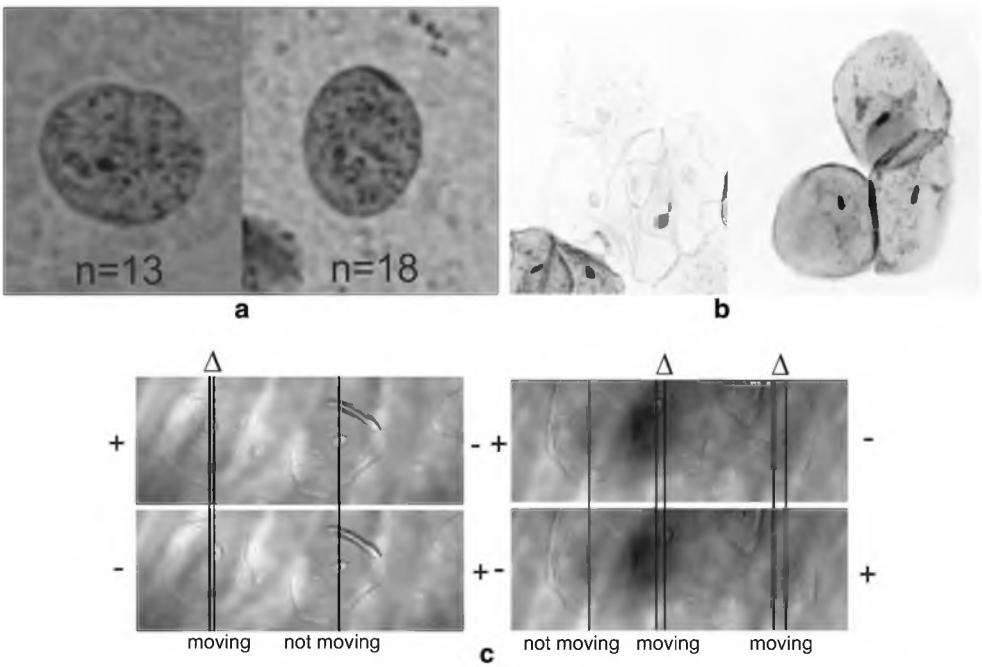

Fig. 2 Images of human buccal epithelial cells: **a** evaluation of the heterochromatin granule quantity [cell nucleus, a control sample (*left*), a sample incubated with one of the drugs (*right*)]; **b** evaluation of cell membrane permeability [cells not stained (*left*) and stained (*right*) with indigo carmine]; **c** evaluation of the cell nuclei electronegativity [nuclei shifting, anode on the *left hand side* (*top*), anode on the *right hand side* (*bottom*)]

(5 mM solution for 5 min) (an example of stained cells is given in Fig. 2b) (Shckorbatov et al. 1995). As a control, when human cells were exposed to detergent solutions known to disrupt cell membrane integrity (Triton X-100, Escin), the percentage of stained cells (PSC, %) increased considerably, indicating a direct correlation of this parameter with membrane permeability (Shckorbatov et al. 1995). In the PSC method 300 cells (3 repeats, 100 cells each) in each experiment were examined. Counting of the stained cells was performed under the MICROmed XS-3330 microscope with 400× magnification by a person 'blind' to the exposure conditions.

Evaluation of the electronegativity of the nuclei

Assessment of the nuclei electronegativity (ENN, %) was performed using the method described in Shckorbatov et al. (2002). Human buccal epithelial cells were placed in the microelectrophoresis unit between two coverslips. The microelectrophoresis unit was then filled with the buffer solution and affixed to the microscope stage. The studies were performed using electric fields of 10–12 V cm^{-1}, electric currents of 0.2–0.4 mA and a fixed frequency of 0.1 Hz. The snapshots of the sample were taken during the experiment (at positive and negative voltage peaks) using a CCD camera. Figure 2c illustrates movement of individual nuclei influenced by changes in electrode polarity. The nuclei inside buccal epithelial cells exhibited either a negative charge and moved toward the anode or did not shift in the electric field, thereby not bearing electric charge under these experimental conditions and sensitivity threshold. The ENN quantity was defined as the percentage of cells with negatively charged nuclei (i.e., shifting in the electric field). For each sample, three ENN measurements were made with 100 nuclei analyzed per measurement, and then the mean value was evaluated.

Determination of changes in drug toxicity in the presence of C_{60} fullerene

Then 0.05 ml drug solution and 0.95 ml C_{60}FAS were mixed (received 1×10^{-5} M of DOX or 2×10^{-5} M of EB or PF solutions with 1.32×10^{-4} M of C_{60} fullerene). To obtain the titration solution, 0.2 ml of the drug solutions with 3.8 ml of buffer solution were mixed (received 1×10^{-5} M of DOX or 2×10^{-5} M of EB or PF solutions). The drug-fullerene mixture with different fullerene concentrations was added to the cell suspension (0.5 + 0.5 ml; the initial concentration of C_{60} fullerene was 6.6×10^{-5} M). The cells were then incubated in the prepared solutions for 1 h. The cells that were incubated only in the drug solutions without C_{60} fullerene added were considered the 'positive control;' the cells incubated without the drugs and fullerene were considered the 'negative control.' The changes in the HGQ and ENN parameters in comparison to the controls were investigated for three donors separately. The incubation of the cells was performed at 36 ± 1 °C. The negative control sample was kept at the same temperature.

Statistical analysis

All computations of the mean values and standard errors were made in Microsoft Office Excel and SigmaPlot. The statistical significance of all observed differences between

the means of obtained data and control values was evaluated using Student's t test. The confidence level was taken as $p < 0.05$.

Results and discussion

The biological activity of the selected DNA-binding aromatic drugs in human buccal epithelial cells was evaluated using three different quantitative parameters, i.e., HGQ, which reflects possible changes in the state of cell chromatin; PSC, assessing the state of the cellular membrane; ENN, defined by the electrokinetic properties of cell nuclei.

Individual action of the drugs

The first stage of the experiment was designed to investigate the individual action of each of the tested drugs, i.e., DOX, EB, PF, and C_{60} fullerene, on buccal epithelial cells in order to determine the optimal concentrations and exposure times for the further study of these compounds in combination. Assessment of the electrokinetic properties of nuclei and the chromatin state as well as the cell membrane permeability was carried out based on the changes in ENN, HGQ and PSC relative to the control sample as a function of the concentration of the compound of interest. The exposure time for the cell culture in the presence of the drugs varies from 10 min to 3 h, and the drug concentration ranges from 10^{-8} to 10^{-5} M. The obtained results are presented in the Supplementary materials (tables with experimental data and Student's t-test results) as well as Fig. 3.

Analysis of the individual influence of DOX, EB and PF shows that the PSC percentage for each sample remains constant within the range of statistically meaningful differences for all the concentrations and exposure times studied for all donors. Consequently, none of these drugs affects the barrier function of the cell membrane, and the PSC parameter can be excluded from further investigations. However, the changes in the structure of heterochromatin (the HGQ parameter) and the nuclei electronegativity (the ENN parameter) are apparent. It is considered that the process of chromatin condensation (heterochromatinization) leads to a decrease in the DNA availability for the different enzymes that play regulatory roles in the process of transcription (Martin and Cardoso 2010). It was found that at concentrations of up to 0.01 mM and exposure times from 10 min to 3 h, the general pattern of DOX, EB and PF impacts on the chromatin state, and the nuclei electronegativity of buccal epithelial cells was generally similar. In particular, the data in Fig. 3 demonstrate the increase in the HGQ parameter after 10 min with DOX exposure, whereas the decrease in the ENN parameter was observed for the highest concentration only (i.e., at 5×10^{-6} M). Incubation with EB and PF also results in an HGQ increase for all drug concentrations after 1 h of exposure, but the changes in the ENN parameter in this case occur only at 1×10^{-5} M concentration.

It should be noted that the mechanism of biological action of aromatic BACs, such as DOX, EB and PF, may also be viewed in terms of other processes such as enzyme inhibition, direct membrane action or generation of ROS (for reviews, see Evstigneev 2010; Evstigneev et al. 2013). The studied drugs are similar in their ability to intercalate into DNA, but are very different in their ability to generate ROS or bind with enzymes or membranes. The fact that all of the studied drugs have demonstrated an apparent influence on the state of the chromatin and a negligible effect on the state of the cellular membrane suggests that the primary mode of the drug action in human buccal epithelial cells is likely to be through binding with DNA.

The obtained data allow choosing the optimal conditions for subsequent experiments in the presence of C_{60} fullerene, i.e., the concentrations of 5×10^{-6} M for DOX and 1×10^{-5} M for EB and PF, and the exposure time equal to 1 h. The selected concentrations correspond to the maximal changes of the HGQ and ENN parameters observed within 1 h of exposure and with no significant further changes after 1 h.

Investigation of the effect of C_{60} fullerene on buccal epithelial cells (see Supplementary material, Table 4) showed no statistically meaningful changes in any of the three parameters for all the concentrations and donors studied. This suggests that C_{60} fullerene by itself does not exhibit any noticeable damaging effect on the cell membrane or nucleus under these experimental conditions. These results agree with the widespread opinion that pristine C_{60} fullerene is non-toxic (Andrievsky et al. 2005).

In summary, based on the results of the preliminary experimental stage, it can be concluded that:

1. The effect of the test compounds DOX, EB and PF is exerted at the cell nucleus and chromatin level, but not at the membrane level. Indirectly, this indicates a DNA-dependent mechanism of action of these drugs on the functional state of the cell. Also the results obtained allow further exclusion of the membrane permeability test as a measure of the biological effects of the test drugs in the presence of C_{60} fullerene.
2. The effect of C_{60} fullerene is observed neither at the nucleus level nor at the level of the membrane. First, it allows neglecting the effect of C_{60} fullerene on the cell's functional state from the measurements of the HGQ and ENN parameters in the subsequent study of joint action of the drugs and fullerene. Second, it indirectly indicates the absence of an apparent cell response from possible C_{60} fullerene binding to DNA, hence the possibility, in a first approximation, to

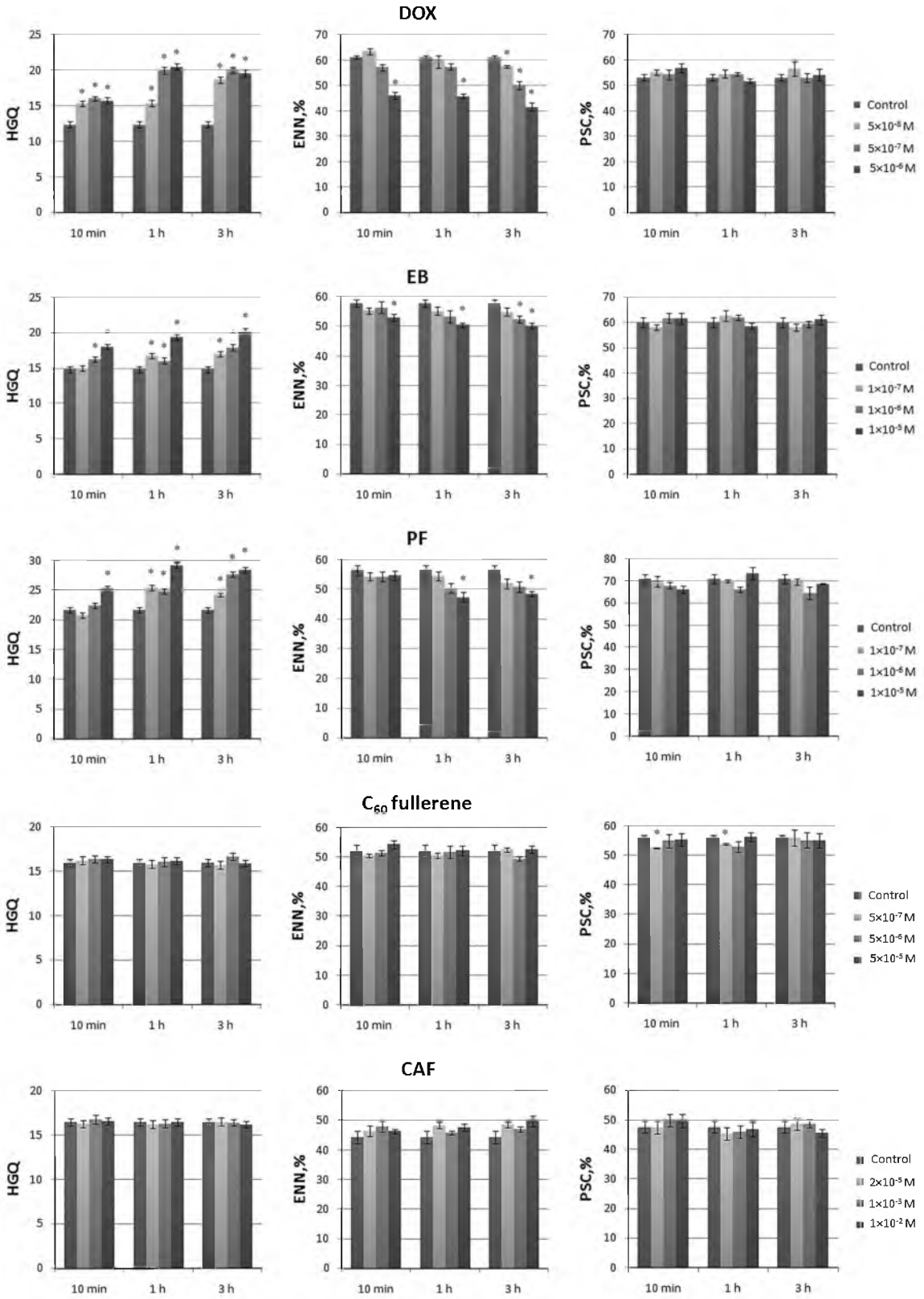

Fig. 3 Changes in the HGQ, ENN and PSC parameters upon exposure to the test compounds: DOX, EB, PF, C_{60} fullerene and CAF (mean values ± standard error; *asterisk* statistically meaningful values with respect to the control; the legend contains the concentrations of the compounds)

exclude the protector mechanism (see the introductory section) from the analysis.

Drug action in the presence of C_{60} fullerene

The results of the experiments for each combination of compounds, viz. DOX-C_{60}, EB-C_{60} and PF-C_{60}, are shown in Fig. 4 (as an example for one donor) and also in the Supplementary material (Tables 6–8).

The most important result that comes from the analysis of the obtained data is the apparent reaction of the cell nucleus to the additional presence of C_{60} fullerene in the system. As in the case of individual action of the drugs, the changes in the measured parameters in the presence of fullerene are qualitatively similar for different drugs and donors. It was established that for each drug a decrease in the HGQ parameter from the positive control level down to the negative control level takes place upon the increase of the C_{60} fullerene concentration, indicating the restoration of the nucleus functional activity by the administration of the C_{60} molecule. The range of the HGQ parameter variation is, on average, two times higher than that for the ENN parameter, indicating higher sensitivity of the heterochromatin restructuring factor as compared with the nuclei electronegativity.

It was also found that the ENN parameter features a high negative correlation (in the range of $r \approx -0.8 \ldots -0.9$) with the HGQ parameter in all performed experiments. The existence of such a relationship between the chromatin state in the nucleus and the nuclei electronegativity for buccal epithelial cells was also reported previously (Shckorbatov et al. 1998), implying the unified nature of the observed effects caused by the combinations of the compounds studied. Our results show that this is not a donor-dependent result.

In the context of the revealed biological synergism in the drug-fullerene systems, we would like to bring attention to the following two issues:

1. The existence of the pronounced dependence of the effects caused by the tested drugs on the C_{60} fullerene concentration (Fig. 4), resulting ultimately in the restoration of the cell system to its initial state;
2. The recently discovered ability of C_{60} fullerene to form stable heterocomplexes with these compounds (Evstigneev et al. 2013).

These features enable us to draw a parallel with the well-investigated drug-xanthine systems (Buchelnikov et al. 2012; Evstigneev et al. 2006, 2008, 2011; Evstigneev 2010, 2013; Goluński et al. 2013; Larsen et al. 1996; Piosik et al. 2010; Traganos et al. 1991; Woziwodzka et al. 2011), for which similar effects are observed on joint in vitro action of aromatic BACs. Of particular importance here is that in the drug-xanthine systems, the operation of the interceptor mechanism (i.e., the drug-xanthine hetero-association) can be considered as widely accepted. Also taking into account that no apparent C_{60} binding with DNA has been reported so far (see the introductory section) and the non-responsiveness of buccal epithelial cells to the addition of C_{60} to the medium observed in our experiments, it is possible to propose that the effects observed in the drug-fullerene systems are mainly described by the interceptor mechanism, while the role of the protector mechanism is negligible. Let us further develop this view.

The published values of the equilibrium constants of hetero-association, K_h, measured under identical experimental conditions for the drug-fullerene and drug-xanthine systems are shown in Table 1. As a typical representative of xanthines and the most well-studied interceptor molecule, caffeine was chosen. The K_h value for the EB-C_{60} system is absent in the literature; therefore, in the subsequent calculations, the intermediate value between DOX-C_{60} and PF-C_{60} constants was used. The reason for this is a significant role of the amino sugar in the structure of the DOX molecule revealed in Evstigneev et al. (2013), which envelopes the surface of C_{60} fullerene and enhances K_h because of the favorable hydrophobic and van der Waals interactions. A similar effect can be expected in the EB molecule as well due to the presence of the phenolic ring, although it has smaller dimensions.

Inspection of the data in Table 1 suggests that the K_h values for the drug-fullerene systems are at least two orders of magnitude higher than the equivalent values in the drug-xanthine ones, giving the reason to expect a more substantial contribution of the interceptor mechanism in these systems as compared with the xanthine-containing ones. Unfortunately, identification of the role of the interceptor mechanism directly at the cellular level is not possible, and in similar works available in the literature, the conclusions about its significance are commonly made on the basis of either the fact of an effective drug-interceptor hetero-association (Piosik et al. 2010; Traganos et al. 1991; Woziwodzka et al. 2011), or the ability to describe in vitro data within the framework of the theory of interceptor/protector action (Buchelnikov et al. 2012; Evstigneev et al. 2008, 2011; Evstigneev 2010). The existence of an effective hetero-association in the drug-fullerene systems has been shown previously (Evstigneev et al. 2013), and the degree of correspondence of the parameters measured in the present work (HGQ, ENN) with the theory of interceptor/protector action was investigated in order to gain further understanding of the role of the interceptor mechanism in the biological effects observed (see Fig. 4).

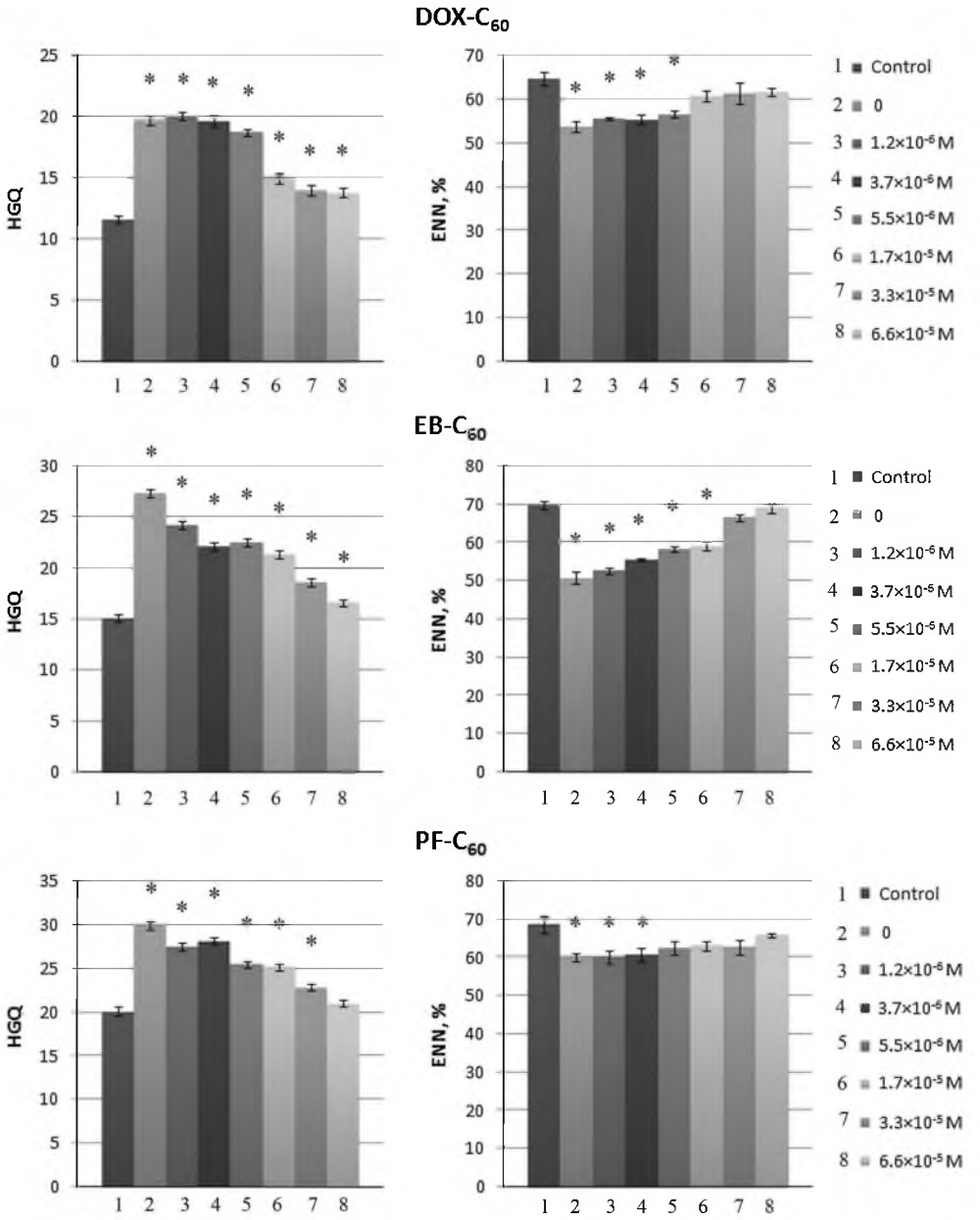

Fig. 4 Changes in the HGQ and ENN parameters upon exposure to the drugs in the presence of C_{60} fullerene (mean values ± standard error; *asterisk* statistically meaningful values with respect to the control; the legend contains the concentrations of C_{60} fullerene)

Table 1 Equilibrium constants of hetero-association (K_h, M^{-1})

	DOX	EB	PF
C_{60} fullerene (Evstigneev et al. 2013)	61,900	44,000*	26,100
CAF (Evstigneev et al. 2008)	208	62	160

* See the explanation in the text

Testing the correspondence of the experimental data to the theory of interceptor/protector action

As a working hypothesis, it was assumed that the interceptor mechanism plays the major role in causing the effects on the chromatin state in buccal epithelial cells in drug-fullerene systems (see Fig. 4). It was also taken into consideration that the drug-fullerene hetero-association induces an additional aggregation of C_{60} fullerene in solution (Evstigneev et al. 2013). Hence, the concentrations of the monomer and the low molecular weight aggregates of C_{60} fullerene in solution can be neglected in a first approximation. Consequently, in a particular case of operation of only the interceptor mechanism in buccal epithelial cells, the theory of interceptor/protector action must predict the concentration dependencies of the measured parameters close to the experimental ones (see Fig. 4). The approximate equation that can describe the experimental data shown in Fig. 4 is presented below.

The theory of interceptor/protector action (Buchelnikov et al. 2012; Evstigneev et al. 2006, 2008, 2011; Evstigneev 2010) uses the system of mass balance equations in the drug-interceptor-DNA system in order to compute the A_D factor, which is proportional to the amount of the drug X displaced from the DNA because of the introduction of the interceptor Y, $f^X_{C(0)} - f^X_C$, relative to the fraction of X-DNA complexes in the absence of Y, $f^X_{C(0)}$ (i.e., at $K_h = 0$)

$$A_D = \frac{f^X_{C(0)} - f^X_C}{f^X_{C(0)}}, \tag{1}$$

where the mole fraction, f^X_C, is defined as $f^X = \frac{K_{XN} x_1 N_1}{x_0}$; K_{XN} is the equilibrium constant of complexation of X with DNA; x_1 and x_0 are the concentration of monomer X and the total concentration of X, respectively; N_1 and N_0 are the concentration of the free X-binding sites on DNA and the total concentration of the X-binding sites, respectively.

It is assumed that the A_D factor is proportional to the change in a biological parameter measured in vitro relative to the control value (in our case, these are the HGQ or ENN parameters) (Buchelnikov et al. 2012; Evstigneev et al. 2006, 2008, 2011; Evstigneev 2010). Once the magnitudes of x_1 and N_1 are known from the solution of the system of mass balance equations, the value of A_D can be further computed and compared against the experimental data. The system of mass balance equations, assuming the absence of the protector mechanism (i.e., the Y binding with DNA), potentially low concentrations of the free drug, x_1 (i.e., not bound to DNA or fullerene) and 1:1 stoichiometry of X–Y hetero-association, is given by

$$\begin{cases} x_0 = x_1 + K_h x_1 y_1 + K_{XN} x_1 N_1 \\ y_0 = y_1 + K_h x_1 y_1 \\ N_0 = N_1 + K_{XN} x_1 N_1 \end{cases}, \tag{2}$$

where y_1 corresponds to the concentration of the free (not bound with the drug) and y_0 is the total amount of C_{60} fullerene.

It is known that the total concentration, N_0, of the available binding sites on DNA is relatively low [about 10^{-6}–10^{-5} M, see (Evstigneev et al. 2008, 2011)], whereas the concentration of C_{60} fullerene, y_0, used in experiments is higher (about 10^{-5}–10^{-4} M). At the same time, the affinities of the drugs to DNA and to C_{60} fullerene are comparable by an order of magnitude, viz. $K_h \sim K_{XN} \sim 10^4$–$10^5$ M^{-1} (Evstigneev et al. 2008, 2013). This implies that a significantly greater portion of the drug X forms complexes with C_{60} fullerene in comparison with the fraction of X-DNA complexes; hence, the $K_{XN} x_1 N_1$ term in Eq. (2) can, in a first approximation, be neglected. In addition, the concentrations of the drugs used in the experiments are sufficiently small ($x_0 \sim 10^{-6}$–10^{-5} M); thus, $K_h x_1 << 1$. The set of these assumptions allows us to reduce the system in Eq. (2) to the form:

$$N_0 \approx N_1,\ x_1 \approx \frac{x_0}{1 + K_h y_1},\ y_0 \approx y_1.$$

Taking Eq. (1) further into account, an approximate expression for the theoretically predicted magnitude of the A_D factor can be written in the form:

$$A_D \approx \frac{K_h y_0}{1 + K_h y_0}. \tag{3}$$

Estimation of the experimental A_D factor can be performed following the approach suggested in Evstigneev et al. (2008, 2011), i.e., by measuring the changes in the biological parameters in the presence of C_{60} fullerene relative to the values of the same parameters in the absence of the fullerene for the same drug concentrations. Since the HGQ parameter was shown to be the most sensitive to any changes in the functional state of the cell (see above), only this parameter was used for the A_D factor evaluation (see Supplementary material) according to the formula

$$A_D = \frac{\frac{N_c - N_0}{N_0} - \frac{N_i - N_0}{N_0}}{\frac{N_c - N_0}{N_0}} = \frac{N_c - N_i}{N_c - N_0}, \tag{4}$$

where N_c and N_0 are the magnitudes of the HGQ parameter in the positive and negative controls, respectively; N_i is the magnitude of the HGQ parameter for the given drug and C_{60} fullerene concentrations.

Figure 5 presents experimental [re-calculated using Eq. (4)] and theoretical [calculated according to Eq. (3)] values of the A_D factor. The results show a reasonably good reproduction of the experimental data when the set of assumptions introduced above within the framework of the theory of interceptor/protector action is used, being especially good for the DOX-C_{60} system ($R^2 = 0.85$). The systematic underestimation of the A_D factor by 10–20 % in EB/PF-C_{60} systems may be considered acceptable, given the absence of any adjustable parameters in the analysis and difficulty in estimating the real values of the concentrations and complexation constants in the intracellular environment. However, we consider that the most important result here is actually not the degree of the coincidence between the theoretical and experimental curves, but the fact that using the sole assumption of hetero-association as the major operating process, and using just the hetero-association constants and the concentrations of C_{60} fullerene, it has become possible to predict a similar order of magnitude effect of recovery of the functional activity of the cell nucleus compared to that observed in the experiment.

Consequently, it can be argued that the initial working hypothesis should be valid, i.e., that the interceptor mechanism (i.e., drug-fullerene hetero-association) plays the

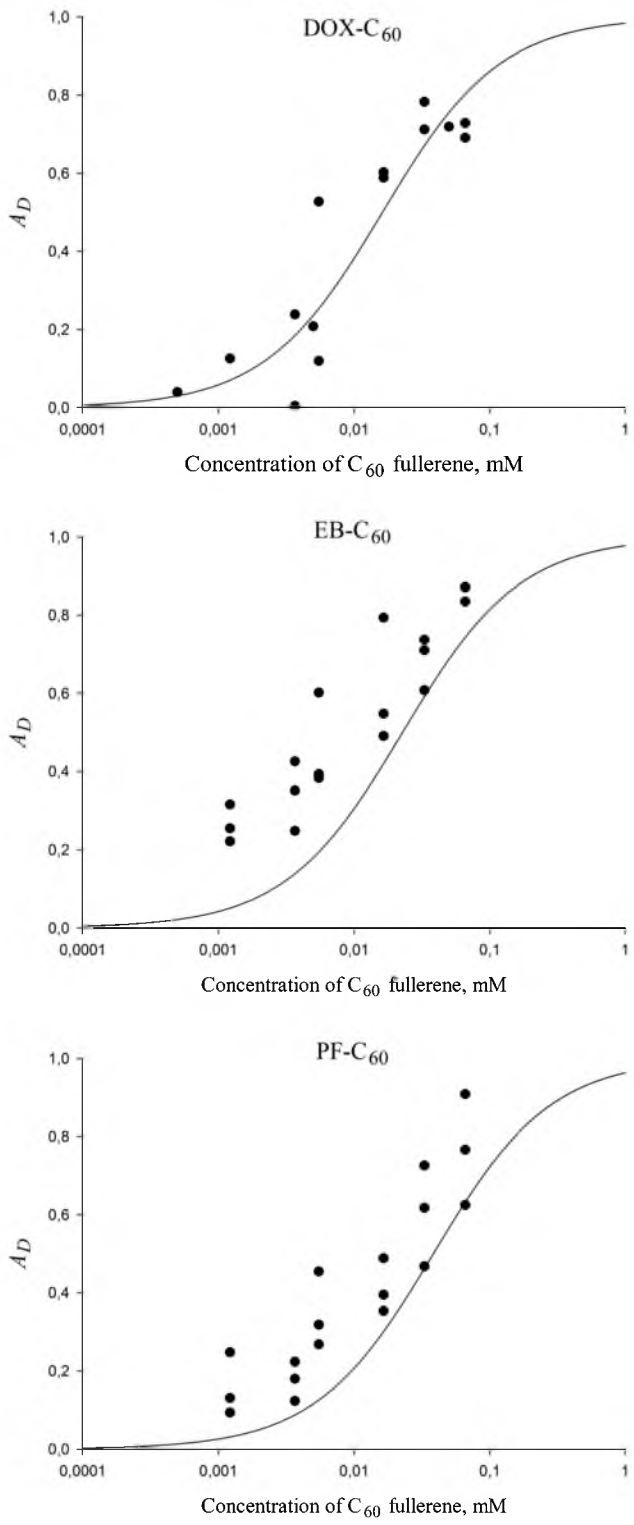

Fig. 5 The A_D factor in drug-fullerene systems as a function of C_{60} fullerene concentration [experimental A_D factor for three donors (*dots*)]; theoretical A_D factor [*solid curve* DOX-C_{60} fullerene: $K_h = 61.9 \times 10^3$ M^{-1} (Evstigneev et al. 2013); EB-C_{60} fullerene: $K_h = 44 \times 10^3$ M^{-1} (see the text); PF-C_{60} fullerene: $K_h = 26.1 \times 10^3$ M^{-1} (Evstigneev et al. 2013)]

dominant role in the observed effects of the action of aromatic BACs on human buccal epithelial cells in the presence of C_{60} fullerene. This statement does not, however, exclude the contribution from other possible mechanisms not discussed in this article, but their role appears to be of minor importance.

The formulated conclusions about the dominant role of the interceptor mechanism are the main outcome of the present work supporting the view that in the observed case of regulation of the in vitro biological activity of aromatic compounds in the presence of C_{60} fullerene, the key role is played by the physico-chemical parameters of the non-covalent interaction between the drug and C_{60} fullerene. In fact, the knowledge of the equilibrium constants of the hetero-association of C_{60} fullerene with different BACs measured in a simple physico-chemical experiment enables us to predict the effect of C_{60} fullerene on the biological activity of aromatic drugs in vitro. Let us further discuss these findings against the extensively investigated combinations of aromatic BACs with xanthine interceptor molecules available in the literature to date.

Effect of the drugs in the presence of CAF

As mentioned above, the concept of interceptor action was previously successfully applied with respect to drug-xanthine combinations in various proliferating cell systems, of which the largest volume of published materials relates to the typical xanthine molecule, CAF [see (Evstigneev 2013; Woziwodzka et al. 2013) for reviews]. Keeping in mind that hetero-association is a molecular process not depending on the type of cell system and assuming that the interceptor mechanism actually dominates in the observed in vitro biological effects in the drug-fullerene systems studied, we can expect to observe a similar biological response (represented by similar changes in HGQ and ENN parameters) in the drug-C_{60} fullerene and drug-CAF systems for the same set of drugs, provided that other experimental conditions are kept identical. In the present work, this hypothesis was tested in the study of the combined action of the drug-CAF systems in buccal epithelial cells.

Analysis of the HGQ, ENN and PSC parameters under the individual action of CAF in the absence of drugs (see Fig. 3 and Supplementary material) demonstrates no statistically significant relationship with the exposure time varying from 10 min to 3 h and concentrations reaching a millimolar range. Consequently, within the limitations of the methods used, CAF itself does not exert a pronounced effect on the functional state of buccal epithelial cells, which is similar to the report above for C_{60} fullerene. This allows evaluation of the action of the drug-CAF combinations to be carried out based on analysis of HGQ and ENN

Table 2 Theoretical and experimental A_D factors in drug-CAF systems

	A_{Dexp}			A_{Dtheor} (interceptor and protector mechanisms)	A_{Dtheor} (interceptor mechanism only)
	Donor A	Donor B	Donor C		
DOX-CAF	0.89 ± 0.17	0.85 ± 0.11	0.62 ± 0.11	0.35	0.18
EB-CAF	0.63 ± 0.15	0.58 ± 0.09	–	0.46	0.14
PF-CAF	0.86 ± 0.17	0.59 ± 0.14	0.55 ± 0.13	0.71	0.44

changes assuming that the changes are only caused by the drug.

The published data indicate that the effects of interceptor action of CAF toward the studied drugs DOX/PF/EB are the most pronounced at the millimolar concentrations of the interceptor molecules (Evstigneev 2013; Traganos et al. 1991). It should also be noted that in case of the drug-CAF systems, the protector mechanism may also be involved (Buchelnikov et al. 2012; Evstigneev et al. 2006, 2008, 2011; Evstigneev 2013); however, its actual role and significance have still not been fully investigated (Goluński et al. 2013; Buchelnikov et al. 2012). Table 2 lists the values of the A_D factors, calculated for the maximal concentration of CAF used in the work ($y_0 \approx 5$ mM), i.e., the experimental one [re-calculated from the measured values of the HGQ parameter using Eq. (4)] and the theoretical one (calculated from the solution of the full system of equations from the theory of interceptor/protector action (Evstigneev et al. 2008), which takes the protector mechanism into account). There is a satisfactory agreement between the theoretical predictions and experimental results. It is also seen that exclusion of the protector mechanism for CAF worsens the quality of agreement between the theory and the experiment, as might be expected. Hence, the initial hypothesis about the dominance of the interceptor and protector mechanisms in nonproliferating human buccal epithelial cells under the action of drug-CAF combinations appears to be valid and provides an additional support to the above-formulated conclusion on the interceptor mechanism of action of C_{60} fullerene on the in vitro biological activity of aromatic compounds.

Conclusion

In the present work, a concentration-dependent restoration of the non-disturbed biological state of the living cell exposed to various aromatic compounds was observed on addition of pristine C_{60} fullerenes by means of assessment of the heterochromatin structure in the cell nucleus and barrier function of the cell membrane and the method of cell microelectrophoresis in buccal epithelial cells. The optimal conditions for the observation of this effect, i.e., exposure time and concentrations of the drugs, were established. The experimental dependence of the number of heterochromatin granules on the concentration of C_{60} fullerene for all donors in the drug-C_{60} systems tested has been sufficiently well described within the framework of the theory of interceptor/protector action (Buchelnikov et al. 2012; Evstigneev et al. 2006, 2008, 2011; Evstigneev 2010), which assumes the dominance of the so-called interceptor mechanism, i.e., non-covalent complexation (hetero-association) of the drug with C_{60} fullerene leading to the observed biological effects. An independent verification of the results was carried out against the drug-caffeine systems, most of which are well described in the literature within the framework of the interceptor/protector action.

In summary, the obtained data indicate the existence of a molecular process (i.e., hetero-association) that does not depend on the type of cell culture used and modulates the in vitro biological effects of the aromatic drugs in the presence of C_{60} fullerene as the interceptor molecule. This result is important in the context of the potential use of pristine C_{60} fullerene together with the aromatic antitumor drugs in combination anticancer therapy.

References

Andrievsky G, Klochkov V, Derevyanchenko L (2005) Is the C_{60} fullerene molecule toxic?! Fuller Nanotub Carbon Nanostr 13(4):363–376

Anilkumar P, Lu F, Cao L et al (2011) Fullerenes for applications in biology and medicine. Curr Med Chem 18(14):2045–2059

Aschberger K, Johnston HJ, Stone V et al (2010) Review of fullerene toxicity and exposure—appraisal of a human health risk assessment, based on open literature. Regul Toxicol Pharmacol 58(3):455–473

Buchelnikov AS, Santiago AH, Flores MG, Ramirez RV, Davies D, Evstigneev M (2012) General analysis of competitive binding in drug–interceptor–DNA systems. Eur Biophys J 41(3):273–283

Buchelnikov A, Kostyukov V, Yevstigneev M, Prylutskyy YI (2013) Mechanism of complexation of the phenothiazine dye methylene blue with fullerene C_{60}. Russ J Phys Chem A 87(4):662–667

Bulavin L, Adamenko I, Prylutskyy Y et al (2000) Structure of fullerene C_{60} in aqueous solution. Phys Chem Chem Phys 2(8):1627–1629

Cataldo F, Da Ros T (2008) Medicinal chemistry and pharmacological potential of fullerenes and carbon nanotubes. Springer, Netherlands

Evstigneev MP (2010) DNA-binding aromatic drug molecules: physico-chemical interactions and their biological roles. LAP. ISBN 978-3-8433-5389-2

Evstigneev MP (2013) Physicochemical mechanisms of synergistic biological action of combinations of aromatic heterocyclic compounds. Org Chem Int. doi:10.1155/2013/278143
Evstigneev M, Khomich V, Davies D (2006) Complexation of anthracycline drugs with DNA in the presence of caffeine. Eur Biophys J 36(1):1–11
Evstigneev M, Lantushenko A, Evstigneev V, Mykhina YV, Davies D (2008) Quantitation of the molecular mechanisms of biological synergism in a mixture of DNA-acting aromatic drugs. Biophys Chem 132(2):148–158
Evstigneev M, Mosunov A, Evstigneev V, Parkes H, Davies D (2011) Quantification of the interceptor action of caffeine on the in vitro biological effect of the anti-tumour agent topotecan. Eur Biophys J 40(8):969–980
Evstigneev MP, Buchelnikov AS, Voronin DP et al (2013) Complexation of C_{60} fullerene with aromatic drugs. Chem Phys Chem 14(3):568–578
Foley S, Crowley C, Smaihi M et al (2002) Cellular localisation of a water-soluble fullerene derivative. Biochem Biophys Res Commun 294(1):116–119
Goluński G, Woziwodzka A, Iermak I, Rychlowski M, Piosik J (2013) Modulation of acridine mutagen ICR191 intercalation to DNA by methylxanthines—analysis with mathematical models. Bioorg Med Chem 21:3280–3289
Kolosnjaj J, Szwarc H, Moussa F (2007) Toxicity studies of fullerenes and derivatives. Bio-applications of nanoparticles, Landes Bioscience and Springer Science, pp 168–180
Larsen RW, Jasuja R, Hetzler RK, Muraoka PT, Andrada VG, Jameson DM (1996) Spectroscopic and molecular modeling studies of caffeine complexes with DNA intercalators. Biophys J 70(1):443–452
Martin RM, Cardoso MC (2010) Chromatin condensation modulates access and binding of nuclear proteins. FASEB J 24:1066–1072
Mchedlov-Petrossyan N, Klochkov V, Andrievsky G, Ishchenko A (2001) Interaction between colloidal particles of C_{60} hydrosol and cationic dyes. Chem Phys Lett 341(3):237–244
Piosik J, Wasielewski K, Woziwodzka A, Śledź W, Gwizdek-Wiśniewska A (2010) De-intercalation of ethidium bromide and propidium iodine from DNA in the presence of caffeine. Central Eur J Biol 5(1):59–66
Prilutskyy YI, Durov S, Yashchuk V et al (1999) Theoretical predictions and experimental studies of self-organized C_{60} nanoparticles in water solution and on the support. Eur Phys J D Atomic Mol Opt Plasma Phys 9(1):341–343
Prylutska S, Matyshevska O, Golub A et al (2007a) Study of C_{60} fullerenes and C_{60}-containing composites cytotoxicity in vitro. Mater Sci Eng 27(5–8):1121–1124
Prylutska S, Matyshevska O, Grynyuk I, Prylutskyy YI, Ritter U, Scharff P (2007b) Biological effects of C_{60} fullerenes in vitro and in a model system. Mol Cryst Liq Cryst 468(1):265–274
Prylutska S, Grynyuk I, Matyshevska O, Prylutskyy YI, Ritter U, Scharff P (2008) Anti-oxidant properties of C_{60} fullerenes in vitro. Fuller Nanotub Carbon Nanostr 16(5–6):698–705
Prylutska S, Burlaka A, Prylutskyy YI, Ritter U, Scharff P (2011a) Pristine C_{60} fullerenes inhibit the rate of tumor growth and metastasis. Exp Oncol 33:162–164
Prylutska S, Burlaka A, Klymenko P et al (2011b) Using water-soluble C_{60} fullerenes in anticancer therapy. Cancer Nanotechnol 2(1–6):105–110
Prylutska S, Burlaka A, Prylutskyy YI (2011c) Comparative study of antitumor effect of pristine C_{60} fullerenes and doxorubicin. Biotechnol 4(6):82–87
Prylutska S, Bilyy R, Overchuk M et al (2012) Water-soluble pristine fullerenes C_{60} increase the specific conductivity and capacity of lipid model membrane and form the channels in cellular plasma membrane. J Biomed Nanotechnol 8(3):522–527
Prylutskyy YI, Durov S, Bulavin L et al (2001) Structure and thermophysical properties of fullerene C_{60} aqueous solutions. Int J Thermophys 22(3):943–956
Prylutskyy YI, Buchelnikov AS, Voronin DP et al (2013) C_{60} fullerene aggregation in aqueous solution. Phys Chem Chem Phys 15(23):9351–9360
Qiao R, Roberts AP, Mount AS, Klaine SJ, Ke PC (2007) Translocation of C_{60} and its derivatives across a lipid bilayer. Nano Lett 7(3):614–619
Scharff P, Risch K, Carta-Abelmann L et al (2004) Structure of C_{60} fullerene in water: spectroscopic data. Carbon 42(5):1203–1206
Shckorbatov YG, Shakhbazov VG, Bogoslavsky AM, Rudenko AO (1995) On age-related changes of cell membrane permeability in human buccal epithelium cells. Mech Ageing Dev 83(2):87–90
Shckorbatov YG, Shakhbazov VG, Gorenskaya OV (1998) The effect of hormones on the electrokinetic properties of the nuclei of human cells. L'Eurobiol 32(234):172
Shckorbatov YG, Shakhbazov VG, Navrotskaya VV et al (2002) Application of intracellular microelectrophoresis to analysis of the influence of the low-level microwave radiation on electrokinetic properties of nuclei in human epithelial cells. Electrophoresis 23(13):2074–2079
Shckorbatov Y, Pasiuga V, Kolchigin N, Batrakov D, Kazansky O, Kalashnikov V (2009a) Changes in the human nuclear chromatin induced by ultra wideband pulse irradiation. Central Eur J Biol 4(1):97–106
Shckorbatov YG, Pasiuga VN, Kolchigin NN et al (2009b) The influence of differently polarised microwave radiation on chromatin in human cells. Int J Radiat Biol 85(4):322–329
Traganos F, Kapuscinski J, Darzynkiewicz Z (1991) Caffeine modulates the effects of DNA-intercalating drugs in vitro: a flow cytometric and spectrophotometric analysis of caffeine interaction with novantrone, doxorubicin, ellipticine, and the doxorubicin analogue AD198. Cancer Res 51(14):3682–3689
Woziwodzka A, Gwizdek-Wiśniewska A, Piosik J (2011) Caffeine, pentoxifylline and theophylline form stacking complexes with IQ-type heterocyclic aromatic amines. Bioorg Chem 39(1):10–17
Woziwodzka A, Goluński G, Piosik J (2013) Heterocyclic aromatic amines heterocomplexation with biologically active aromatic compounds and its possible role in chemoprevention. ISRN Biophys 740821:11. doi:10.1155/2013/740821
Zhao X, Striolo A, Cummings PT (2005) C_{60} binds to and deforms nucleotides. Biophys J 89(6):3856–3862