Nucleotide carriers for anti-tumour actinomycin antibiotics

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We have investigated a number of complexes of 7-aminoactinomycin D (7AAMD), with its potential carriers: caffeine, folic acid (FA), purine bases—guanine and adenine, pyrimidine base—thymine and with fragmented DNA to determine more stable and suitable complex. The process of binding of the fluorescent antibiotic with clusters of caffeine, guanine, adenine, thymine and with fragmented DNA was accompanied by a considerable long-wavelength shift in excitation spectrum. The energy of interaction between phenoxazine hetero-cycle of 7AAMD and chromophores of the carriers studied has been found. In the case of 7AAMD with guanine, adenine, thymine and caffeine, the energy is about of 7 kcal/mol, which is a little lower than in the case with DNA (7.7 kcal/mol). On the basis of emission spectra, in all examined compounds, with the exception DNA, the 7AAMD molecule emits photons from water phase, not from a cluster, since photo-excitation leads to desorption of the antibiotic from a cluster surface. We observed also the mutual fluorescence quenching of 7AAMD and FA in their complex. It may well be that this complex forms due to interaction of peptide-lactone rings of 7AAMD with system of FA. In the case of DNA, the complex with 7AAMD has very high stability that is determined not only by interaction between phenoxazine of 7AAMD and the DNA bases, but it is largely owing to the interaction between two peptide-lactone rings of 7AAMD and the DNA deoxyribose-phosphate chains.

Keywords: 7-aminoactinomycin/actinomycin/caffeine/fluorescence spectroscopy/fragmented DNA.

Abbreviations: 7AAMD, 7-aminoactinomycin D; AMD, Actinomycin D; FA, folic acid.

Actinomycin D (AMD) is one of the most effective natural anti-cancer antibiotics. Its structure contains a phenoxazine hetero-cycle and two peptide-lactone rings (1, 2) (Fig. 1). Blockade of the RNA polymerase reaction in DNA give rise to high anti-tumour activity, with leading to termination of a protein synthesis and cell division (3–5).

There are some constraints to the use actinomycin in medicine. The most important is the toxic effect of AMD to normal cells at its high concentrations. We

are supposed to reduce this toxic side effect by using a number of carriers used in our work. Moreover, we have prepared a complex with folic acid (FA) for individual therapy for some types of cancer, which characterized over-expression of folic receptors on the surface of cancer cells.

It has been known, that in DNA solutions, the AMD molecules at physiologically low concentrations incorporate into unwound DNA regions with the non-stacking mechanism (6–9). AMD is a large molecule, which at low concentrations poorly binds in a stacking-like manner to the classical DNA double helix (it takes place only at high concentrations). Also, AMD badly penetrates into cells across a plasma membrane. Therefore, special carriers should be used to enhance the penetration of the antibiotic into tumour cells. Unfortunately, a number of attempts to deliver AMD into tumour cells were not too successful even by using phospholipid liposomes, polypeptides, antibodies, synthetic polymers and surfactants (10).

According to some data, which have been obtained in our laboratory, the actinomycin antibiotics penetrate more easily into tumour cells together with the hairpin oligonucleotide HP1 and caffeine (11). It was shown by using 7-aminoactinomycin D (7AAMD), a fluorescent analogue, which is well detected in micromolar concentrations. HP1 and caffeine applying allow the therapeutic concentration of the antibiotic to be decreased, and the anti-tumour action to be enhance simultaneously (12). Antibiotic binds with HP1 hairpin or with caffeine clusters surface, which allows improved penetration through membranes. An enhanced penetration of AMD and 7AAMD in complex with HP1 and caffeine was observed on model carcinoma cells (12). It is important that the hairpin HP1 and caffeine do not prevent the transferring of antibiotic from one to DNA (13).

Synthesis of hairpin oligonucleotides is a laborious process, and their biological action is not always predictable. Therefore, caffeine and its analogues may become the most convenient carriers for antibiotics (14). Nucleotides such as adenine, guanine, thymine and cytosine are intensively consumed during proliferation of rapidly dividing DNA in transformed cells. This completes the selective delivery of the antibiotic predominantly to tumour cells. Probably, they should give the best action for potential treatment.

Other component of the carrier particles could be FA. The FA molecule consisted of two parts: the p-aminobenzamide system and the 2-amino-4-hydropteridine system. The pteridine part is similar to purine and pyrimidine nucleotides. The p-aminobenzamide part is a bit similar to glutamine acid. Due to these two factor FA can be consumed in high quantities during cell proliferation and that is why FA easily penetrates into cells by natural membrane-binding

Fig. 1 Excitation spectrum of 2 μ M 7AAMD in aqueous solution (1), in the 2 mM adenine solution (2), and (3) is the difference.

folic transporters. Thus, FA could serve as a precursor molecule.

Hetero-association of AMD with caffeine at their milli-molar concentrations was studied by NMR spectroscopy (15). However, such high concentrations of AMD are not physiological. Moreover, the authors of Ref. (15) did not take into account that caffeine and AMD at milli-molar concentrations in aqueous solutions which do not exist as monomers but form aggregates or ordered clusters (16–19).

It is necessary to work with smaller amounts of the antibiotic and to take into account the presence of aggregates of caffeine or its analogues. Recently in our lab, the energy of interaction of chromophore of AMD with nucleotides in solution was determined by spectrophotometry method (6). The goal of this study is to investigate by sensitive spectrofluorimetry method the interaction of 7AAMD at low (micro-molar, physiological) concentrations with adenine and guanine aggregates, caffeine clusters, FA and fragmented DNA to determinate the most suitable carrier.

Thus, in this article, we propose a variety of carriers to reduce the toxic effect of the antibiotic actinomycin. Proposed vectors (purine base, thymine, caffeine, FA) are promising compounds for use as carriers, when they are rapidly consumed in large amounts by malignant cells. Moreover, there are some folate-specific receptors expressed on that certain types of cancer. There is a reason to assume specific antibiotic delivery to cancer cells, using the carriers proposed here. In this article we show the formation of antibiotic-carrier complexes using highly sensitive fluorescence spectroscopy. We illustrate a fluorescent screening of carriers, which allow to identify the most suitable carrier, according to the

value of the interaction energy. It seems the most suitable candidate for use as a carrier is caffeine, because it has one of the highest energies of interaction with actinomycin, wherein it is well soluble in water and has an affinity to lipids, which allow it to penetrate easily into the cell remaining actinomycin molecules.

Experimental Procedures

Reagents

Adenine, guanine, thymine (Fluka), AMD (Reanal), caffeine (Panreac), FA (Sigma), 7AAMD and fragmented salmon sperm DNA (Sigma) were used. The concentrations of adenine, guanine, caffeine, thymine, FA and nucleotides of fragmented DNA in experiments were from 2×10^{-4} to 2×10^{-3} M (in distilled water). Fragmented DNA was dissolved in distilled water and prepared the solution of 2×10^{-3} M per nucleotides. 7AAMD was added at a very low concentration, 2×10^{-6} M. The fluorescence measurements were done 20–30 min after mixing.

Spectroscopy

Absorption spectra were recorded on a Cary-100 spectrophotometer in cuvettes with optical path from 0.1 to 1 cm in the ranges of 220–300 nm (the absorption band of nucleotides) and 470–570 nm (the absorption band of 7AAMD) at room temperature. The excitation and emission spectra of 7AAMD and FA were measured with an updated Perkin-Elmer MPF44B spectrofluorimeter.

Energy of interaction

The energy of interaction between chromophore of the antibiotic and a carrier was determined by means of difference spectra obtained by subtracting the excitation spectrum of 7AAMD from the excitation spectrum of a mixture of 7AAMD with purines, caffeine, thymine, FA or DNA. First, a small contribution of light scattering on purine clusters or native DNA was subtracted from the spectrum of the mixture in the visible region, then the normalization at the maximum to spectrum of a free 7AAMD in water was done, and after that the difference of the two spectra was taken. Second, the normalization procedure makes it possible to eliminate a

contribution to fluorescence intensity from the decrease in the molar absorption coefficient and a contribution from oppositely directed enhancement of fluorescence quantum yield of 7AAMD at incorporation into aggregates, clusters or DNA. Besides, the normalization eliminates possible inaccuracies in addition of substances by a micro-pipette and a partial adsorption of the antibiotic onto a surface of a plastic pipette tip. The energy of interaction between chromophores in complexes was determined by the well-known relation $E = h \Delta\nu$ where h is the Planck constant, and $\Delta\nu$ is the spectral shift in the scale of wave numbers (in cm^{-1}).

Results and Discussion

Aggregates and clusters

It has been recognized that guanine and adenine form aggregates in distilled water, although in not too high concentration, about of 10^{-5} M (16, 19). Caffeine and thymine at milli-molar concentrations form small ordered aggregates—clusters, consisting of 10–20 molecules (14–19). The aggregation results from hydrophobic interactions and hydrogen bonding. The size of a small aggregate or cluster is about of 50–150 Å this is many times less the wavelength. Therefore, upon the formation of such small aggregates or clusters, the solution remains transparent (the turbidity is negligibly small). The caffeine, adenine, thymine and guanine concentrations, used in our study, were limited. The quantity of nucleotide molecules was taken hundred times greater than quantity of 7AAMD molecules. A further increase in the nucleotide concentration would hardly change the interaction energy, but will lead to precipitation. At aggregation of guanine, adenine, thymine and caffeine, their molar absorption coefficient at 260 nm was decreased (due to the screening hypochromism effect—a competition for the photon in a stack-arranged hetero-cycles (17), and also due to light scattering on large aggregates), but no new band appeared. This means that the energy of interaction between molecules in aggregates is low.

Interaction of 7AAMD with adenine

Two methyl groups of phenoxazine hetero-cycle allow the antibiotic to be adsorbed on a surface of adenine cluster due to hydrophobic interactions. Addition of 7AAMD at a concentration of 2×10^{-6} M to a solution of adenine, taken at a 1,000-fold higher concentration (2×10^{-3} M; at this concentration, adenine molecules are almost completely clusterized) lead to a long-wavelength shift in the excitation spectrum of this fluorescent antibiotic. Thus, phenoxazine chromophore of 7AAMD forms a stable hetero-complex with adenine cluster. At the big ratio of used concentrations, one adenine cluster can bind no more than one 7AAMD molecule, and the most population of clusters stay without 7AAMD. Only one antibiotic molecule is adsorbed onto the surface of a cluster. Fluorescence quantum yield of 7AAMD upon the binding markedly increases, because chromophore of the antibiotic passes from polar liquid water phase to less polar and more solid phase—a cluster surface.

Under these conditions, the excitation spectrum of 7AAMD shows a long-wavelength shift of at least of 20 nm relatively to that of free 7AAMD in water (Fig. 1 and Table I). Also, the shape and the half-width

Table I. Parameters of the excitation spectrum of 7AAMD in purine complexes

Excitation spectrum of 7AAMD	λ_{max} , nm	$H_{1/2}$	$\lambda_{\text{diff max}}$, nm	$\Delta\nu$, cm^{-1}	E , kcal/mol
In water	496	105	—	—	—
+ FA	496	105	—	—	—
+ Guanine	533	110	565	2,460	7.0
+ Adenine	524	110	562	2,370	6.8
+ Caffeine	530	110	563	2,400	6.9
+ Thymine	510	120	557	2,140	6.0
+ Fragmented DNA	549	100	572	2,680	7.7

^aEmission was 670 nm. Concentration of 7AAMD was 2×10^{-6} M, guanine— 10^{-4} M, caffeine, adenine, thymine and DNA (nucleotides)— 2×10^{-3} M; λ_{max} is the wavelength of the excitation spectrum maximum; the accuracy in λ was ± 3 nm; $H_{1/2}$ is the half width of this spectrum; $\lambda_{\text{diff max}}$ is the long-wavelength maximum of the difference spectrum; $\Delta\nu$ is the shift of the excitation spectrum maximum of 7AAMD of the complex in the difference spectrum relative to those in water (440 nm); E is the energy of interaction.

of the spectrum sufficiently change. All these features indicate a significant redistribution of electron density in chromophore of 7AAMD, *i.e.* show antibiotic interaction with adenine chromophores. It should be noted that the excitation spectra, as it is well-known, is similar on shape and position to absorption spectra. They provide information about ‘absorption’ electron transitions though they are detected from fluorescence intensity (the sensitivity of fluorescence method is 1,000 times higher than spectrophotometry).

The energy of interaction between chromophore of 7AAMD and adenine chromophore can be estimated from the spectral shift in the scale of wave numbers (cm^{-1}). The shift in the mixture of 7AAMD with adenine relatively to a free 7AAMD in water was $1,080 \text{ cm}^{-1}$ this corresponds (according to known relation $\Delta E = h \Delta\nu$) to the energy of 3 kcal/mol (the statistical inaccuracy in the energy, calculated from repeating of recorded spectra, was ± 0.3 kcal/mol). This energy reflects only the lower boundary of the energy, because a considerable contribution to the excitation spectrum of 7AAMD in the mixture with adenine belongs to antibiotic molecules that do not bind to adenine tightly. To estimate the most energy of binding more precisely, it is necessary to use a difference spectrum, which is obtained by subtracting the excitation spectrum of 7AAMD in water from the excitation spectrum of 7AAMD in a mixture with adenine (Fig. 1). For this purpose, first, a small contribution of adenine light scattering was subtracted from spectrum of the mixture (in the visible region). Then the normalization at the spectral maximum of free 7AAMD in water was done, and the difference of two spectra was taken. The normalization procedure makes possible to eliminate the oppositely directed contributions to the measured fluorescence intensity of two factors: a decrease in the extinction coefficient, and an increase in the fluorescence quantum yield of 7AAMD upon sorption onto adenine clusters. At maximum at the positive band of the 7AAMD excitation on adenine clusters in the long-wavelength region of

Table II. Parameters of the emission spectrum of 7AAMD in nucleotide clusters

Emission spectrum of 7AAMD	$\lambda_{\max}$, nm	$H_{1/2}$, nm	F
In water	658	69	1
+ FA	658	69	0.5
+ Guanine	658	73	2
+ Adenine	661	78	1.7
+ Caffeine	662	76	2.2
+ Thymine	660	72	1.5
+ Fragmented DNA	627	84	7.8

^aExcitation was 550 nm. Concentration of 7AAMD was 2×10^{-6} M, guanine— 10^{-4} M, caffeine, adenine, thymine and DNA (nucleotides)— 2×10^{-3} M; $\lambda_{\max}$ is the wavelength at the emission maximum; $H_{1/2}$ is the half width of the emission spectrum; F is the intensity of 7AAMD emission (taken as 1 in an aqueous solution).

the difference spectrum lies at 562 nm (Fig. 1). The shift relatively to 7AAMD in water is $2,370 \text{ cm}^{-1}$ that corresponds to the energy of 6.8 kcal/mol. This suggests that the antibiotic molecule is more or less rigidly bound on the adenine cluster.

The difference spectrum has also a negative peak—at 480 nm (Fig. 1). It would follow from basic rule of spectroscopy that the intensity of a new band should be equal to a decrease in the intensity of the initial band (16), we can conclude, judging from the approximate equality of the areas of the positive and negative peaks, that the positive and negative peaks of 7AAMD in adenine clusters are almost entirely induced by inter-chromophore interactions.

After incorporation of 7AAMD into adenine clusters, its fluorescence quantum yield increases two times compared with a free 7AAMD (Table II). However, the 'emission' spectrum has not shown a sufficient shift (Fig. 2). Judging from the position of the emission spectrum, 7AAMD molecules emit photons not from adenine clusters but from aqueous phase. This seems as contradiction to the above conclusion, done from excitation spectra. However, this seeming contradiction can best be understood, if we take into account that photo-excited 7AAMD molecules can be released from the surface of adenine clusters to aqueous phase. The process of photo-desorption occurs in sub-nanosecond time scale (fluorescence life-time of 7AAMD is 1–2 ns). The photo-desorption is due to that the difference in the energies between excitation and emission of 7AAMD is expended for break the bond of 7AAMD with adenine cluster. The photo-excitation leads to release of the antibiotic from the surface of cluster to aqueous phase. The oscillatory (thermal) energy in photo-excited antibiotic is insufficient to destroy the cluster itself (which combines of 10–20 molecules), but is sufficient to destroy 2–3 bonds between one excited antibiotic molecule and 1–2 adenine molecules.

It would be stressed that our method shows the interaction only between chromophores. We cannot estimate directly the contribution of peptide-lactone ring in binding. But, from facts such as those reviewed here, it seems likely that actinomycin binds with

Fig. 2 Emission spectrum of 2 μM 7AAMD in aqueous solution (1) and in the 2 mM adenine solution (2).

surface of adenine clusters. It would be reasonable to suppose that under excitation the chromophore of 7AAMD a bit separates from the surface and illuminates from water phase, with peptide-lactone ring remains bind with cluster surface (Fig. 3).

Interaction of 7AAMD with guanine, caffeine and thymine

Some researchers believed that, among DNA bases, guanine has the maximum affinity for AMD (1, 2). If so, it might be expected that 7AAMD in water solution would readily interact with guanine. Guanine at concentration above 5×10^{-5} M in water exists predominantly in the form of aggregates (16, 19). It is important to note that addition of 7AAMD (in low concentration, 2×10^{-6} M) to a solution of guanine at its 50-fold higher concentration (10^{-4} M) led to formation of hetero-complexes. The addition of 7AAMD in concentration of 2×10^{-6} M to the solution of thymine or caffeine, taken at a 1,000-fold higher concentration (2×10^{-3} M; at this concentration, thymine and caffeine molecules are almost completely clusterized), or guanine, taken at a 50-fold higher concentration, leads, as in the case of adenine, to long-wavelength shift in the excitation spectrum of 7AAMD. The magnitude of the shift, derived from the difference spectrum (Table I) is $2,140 \text{ cm}^{-1}$ in the case of thymine $2,400 \text{ cm}^{-1}$ in the case of caffeine and $2,460 \text{ cm}^{-1}$ in the event of guanine. Hence, it follows that the energy of interaction of chromophore of 7AAMD with thymine, caffeine and guanine is 6.0, 6.9 and 7.0 kcal/mol, respectively. Values of energy of interaction in the case with caffeine and guanine differ negligibly from the value for adenine, 6.8 kcal/mol. This is in agreement with our data (8, 9, 19) and the results of other authors (20–22) indicating that actinomycin antibiotics have not a special high affinity for guanine. So, we can go to conclusion that antibiotic can almost equally well connect with all the studied substances, with a slight increase of the interaction energy in cases of guanine and caffeine. It should be noted, the 7AAMD in our experiments was added to aggregates

Fig. 3 Scheme of complex between the 7AAMD molecule and the surface of cluster.

or clusters. At low (micro-molar) concentrations of caffeine, purines and thymine, when they are not aggregated, the antibiotic does not interact with them: no changes in its fluorescence occur (data are not presented).

Interaction of 7AAMD with FA

The addition of 7AAMD at a concentration of 2×10^{-6} M to a solution of FA taken at a 200-fold higher concentration (4×10^{-4} M; FA above this concentration begin to precipitate) leads to relative

Fig. 4 Excitation spectrum of 400 μM FA in aqueous solution (1a) and with addition of 2 μM 7AAMD (1b), and emission spectrum of 400 μM FA in aqueous solution (2a) and with addition of 2 μM 7AAMD (2b).

fluorescence quenching the both—antibiotic and FA, without any shift in the excitation spectrum (Fig. 4). Despite of the concentration of FA was sufficiently greater than the antibiotic concentration, but the FA fluorescence was significantly quenched in the presence of 7AAMD. Therefore, we suggest the presence of two forms of FA: the fluorescent (minor) and non-fluorescent (major).

Interaction of 7AAMD with fragmented DNA

The use of fragmented DNA (instead of native DNA) in our experiments has two advantages: a low light scattering and a great number of binding sites for the antibiotic. In addition, strongly fragmented DNA, as opposed to the native one, could serve as a carrier of antibiotic into cells.

Fluorescence quantum yield of 7AAMD at binding with DNA abruptly increases (17). The shape of the excitation spectrum of 7AAMD in DNA is considerably changed, and a strong shift to long-wavelength region takes place (Table I). The situation is similar to that happens after addition of 7AAMD to solutions of adenine, guanine etc.; however, the effects are much more pronounced.

The excitation spectrum of 7AAMD in DNA principally differs from that of 7AAMD in water. The shift of the excitation spectrums of 7AAMD (concentration of 2×10^{-6} M) and DNA (concentration is 2×10^{-3} M, nucleotides) was $2,680 \text{ cm}^{-1} \text{ cm}$. Consequently, the energy of interaction between chromophore of 7AAMD and DNA bases is 7.7 kcal/mol. This value is a bit more than values, obtained in the case of purine aggregates and caffeine clusters.

It should be stressed that the 'emission' spectrum of 7AAMD in DNA (as distinct from those with purine aggregates and caffeine clusters) shows a strong 'shift to shorter' wavelengths, and the fluorescence intensity increases considerably (Table II). Judging from the short-wavelength emission spectrum, 7AAMD in DNA emits from the hydrophobic phase rather than

aqueous one (17, 19). Thus, the photoexcited antibiotic does not leave the DNA. At low concentrations, the antibiotic is incorporated into unwound DNA regions without intercalating into the double helix between the planes of nucleotide bases (8, 9, 19). It stabilizes DNA (23), not allowing small unwound regions to become 'starting points' of a larger unwinding. This is why the antibiotic increases the DNA melting temperature (19). It is worth noting that, at very high concentrations, the antibiotic intercalates into double helix (24). However, in this case, it acts as a mutagen, similar to ethidium bromide, which unwinds the DNA double helix.

It should be reemphasized, that our method allows appraise directly only interaction between chromophores. In view of the example just cited, the photoexcitation does not lead to release of the antibiotic from DNA. Consequently, the rigidity of its binding is determined not so much by the interaction between phenoxazine and nucleotides (it almost the same as in the case of adenine, guanine and caffeine clusters). More important, that where is the interaction of peptide-lactone rings with the DNA deoxyribose-phosphate chains. Phenoxazine places inside DNA—in the hydrophobic site of an unwound region.

Unfortunately, most of common methods cannot be applied to solutions of micro-molar concentrations for determination the association energy. Similar results on the energy have been obtained by other fluorescence approach. We determined the association constant for the binding of 7AAMD to caffeine clusters (Fig. 5) and fragmented DNA (data are not presented) and calculated the equivalent energy of interaction. The association constant is $K \approx 3.14 \times 10^5 \text{ (M}^{-1}\text{)}$. Thus, the energy of interaction $\approx 7.6 \text{ kcal/mol}$. It is the practically same result, which we obtained by our simple method (7.8 kcal/mol). The concentration of 7AAMD is much less than one of the caffeine clusters, thus we propose that all molecules of the antibiotic is bind. We obtained results absolutely alike: 7.6 kcal/mol in the case with caffeine and 13 kcal/mol in the case with

Fig. 5 Fluorimetric titration of caffeine clusters by 7AAMD in inverse coordinates (by Scatchard's method (25)).

fragmented DNA. From the proceeding discussion it is clear that the obtained value in the case with DNA shows the energy part of peptide-lactone contribution to binding.

The obtained data on the interaction of chromophore of 7AAMD with fragmented DNA is in accordance with ones for native DNA (19, 23, 26–29) but are more convincing, since fragmented DNA has more binding sites than the native DNA.

Thuswise, in this study we proved that despite of actinomycin antibiotics have not high specificity for guanine, however the binding exists and the energy of interaction of phenoxazone of 7AAMD with guanine a bit more, than with adenine, thymine, caffeine and FA. We proposed a simple and sensitive fluorescence method for determining the energy of interaction of the chromophore of heterocyclic antibiotic with potential carriers: purine aggregates, caffeine clusters and fragmented DNA. We showed that the antibiotic can be released by photo-excitation from clusters into the aqueous phase, but it cannot go out from DNA. We demonstrated the difference between mechanisms of interaction of antibiotics with various carriers and fragmented DNA. This allows resolve, where the fluorescent molecule places: inside of a particle or outside. This approach can be applied to a number of studies for variety of biological structures where fluorescent probes are used.

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Conflict of Interest

None declared.

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