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ORIGINAL PAPER

Actinomycins Like Anti-Cancer Photo-Sensitizers

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Abstract Spectroscopic and microscopic study on application of actinomycins as anti-tumor photo-sensitizing drugs was carried out in this work. It has been shown that 7-aminoactinomycin (7AAMD) and actinomycin D (AMD) inside cells of line *HeLa* bind not only with DNA, but also with proteins. Fluorescence of 7AAMD in *HeLa* cells and destruction of these cells by photosensitizing with actinomycin D were detected. When photo-destruction occurs, the antibiotic is released out from destroyed cells.

Keywords Actinomycin D · 7-aminoactinomycin D · Tumor cells *HeLa* · Photo-sensitizing · Fluorescence

Introduction

Antibiotics of actinomycin series are widely used to treat infectious and malignant diseases [1, 2]. This application is based on the ability of actinomycins to form specific stable complexes with DNA, leading to inhibition of RNA polymerase reaction and resulting in suppression of protein synthesis and cell division.

Natural heterocyclic antibiotic actinomycin D (AMD) and its fluorescent derivative 7-aminoactinomycin D (7AAMD) form *at low concentrations* non-stacking complexes, stabilizing the structure of DNA [3–5]. And perhaps that is why they exhibit anti-tumor activity, in contrast to stacking-intercalating dyes, for instance, ethidium bromide—the most powerful carcinogen, destroyed the DNA double helix [5]. At low concentrations, actinomycins block only the mitotic activity of cells, without affecting the basic metabolic processes.

AMD is a typical representative among actinomycins, consisting of a flat phenoxazone chromophore (4,6-dimethyl-2-amino-phenoxazone-3-one-1,9-dicarboxylic acid) and two identical penta-peptide-lacton groups, two amino groups of which are acylated by two carboxyl chromophores.

Incorporation of triated AMD into drug-sensitive and drug-resistant *HeLa* cells was investigated many years ago [6]. 7AAMD as a fluorescent cytochemical probe was firstly applied in [7].

It has been shown that AMD greatly enhances the DNA photosensitization [8]. Superoxide radical anion and radical anion of AMD were identified as intermediates in the photodynamic process. The obtained data were explained as electron transfer from DNA to photo-excited AMD.

Photo-reactions of 7AAMD with and without DNA were studies by fluorescence correlated spectroscopy [5, 9]. It can be suggested that anti-tumor activity of the antibiotic could be dramatically stimulated by light exposing.

In the present work, the spectroscopic and microscopic studies on application of actinomycins as anti-tumor photo-sensitizing drugs are carried out. Fluorescence of 7AAMD in cells of line *HeLa* and photo-induced damage of these cells by illumination of AMD were detected for this aim.

Experimental

Tumor cells *HeLa* were cultured in a nutrient medium RPMI 1640. The job was carried out with suspensions of cells in 20 mM Tris with 200 mM sucrose (pH 7.5). After incubation with AMD or 7AAMD, the samples were centrifuged at 600 rpm for the deposition of cells (in

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Table 1 Fluorescence intensity of 7-amino-actinomycin D in *HeLa* cells

Initially:	Fluorescence intensity (rel.un.)
7AAMD in external solution	0.25
Cells, stained by 7AAMD	0.31
Cells	0.26
After 30-min incubation at 37 °C	
7AAMD in external solution	0.01
Cells, stained by 7AAMD	2.0
Cells	0.66

The samples were tested in mirror micro-cuvettes. Excitation was 540 nm, emission was 610 nm

which, therefore, only intracellular 7AAMD or AMD was contained). Then, the supernatant was discarded, and the precipitate of cells was diluted to the initial concentration.

AMD ("Serva") and 7AAMD ("Fluka") were used. Concentration of 7AAMD in most cases was 2 μM, AMD – 20 μM.

Photo-sensitizing experiments were done by light irradiation from the 450-Wt xenon lamp (with heat filter—copper sulfate solution). The diluted suspension of cells was placed in special mirror cuvette (to increase the irradiation efficiency) [5], supplied with a special thermostatic holder.

Absorbance and fluorescence of AMD and 7AAMD in the mentioned buffer with or without caffeine were detected in 1-cm quartz cells at 20 °C by spectrophotometer "M-40" and spectrofluorometer "SLM-4800". The excited state lifetime of 7AAMD was measured by phase method with "SLM-4800" in mirror cuvettes [5] to increase the fluorescence signal by many times.

Results and Discussion

Penetration of Actinomycins into *HeLa* Cells

7AAMD and AMD at micromolar concentrations under "soft" conditions (5-min incubation at 20 °C) did not penetrate into the *HeLa* cells.

Table 2 Effect of added AMD on the intensity of tryptophan fluorescence of proteins in *HeLa* cells

Samples:	Intensity of tryptophan fluorescence (rel. un.)
Cells	0.14
Cells + AMD	0.07
Cells + AMD + caffeine	0.12

The incubation time was 30 min. Caffeine concentration was 2 mM. The emission was measured after re-precipitation of cells. Excitation was 295 nm, emission was 338 nm

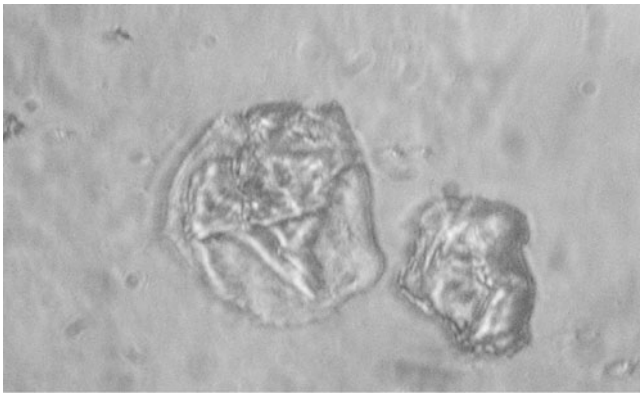

Fig. 1 Photomicrographs of two *HeLa* cells, stained by AMD. Carl Zeiss microscope and camera Sony Cyber Shot (resolution of 5 megapixels) were used

Bad penetration of heterocyclic antibiotics into cells can be improved by heating of cells and prolongation in the time of incubation with antibiotic [5] and also—through formation of special complexes with hairpin oligonucleotides [5, 10], caffeine [5, 11] or lipids [2]. Most simple methods are heating and prolongation.

There was a good penetration of actinomycins under more "hard" conditions: 30-min incubation at 37°C. Table 1 shows the data on incorporation of 7AAMD from external solution into *HeLa* cells. It is clearly seen that the external solution initially contains 7AAMD, but the antibiotic disappears from the solution after such incubation. Almost all molecules of the antibiotic pass into cells. It should be noted the existence of the initial (before incubation) non-additivity of the 7AAMD fluorescence intensity and background intensity of cells. This clearly suggests that 7AAMD initially emits less from cells than from the external solution. So, either it inside cells worse absorbs

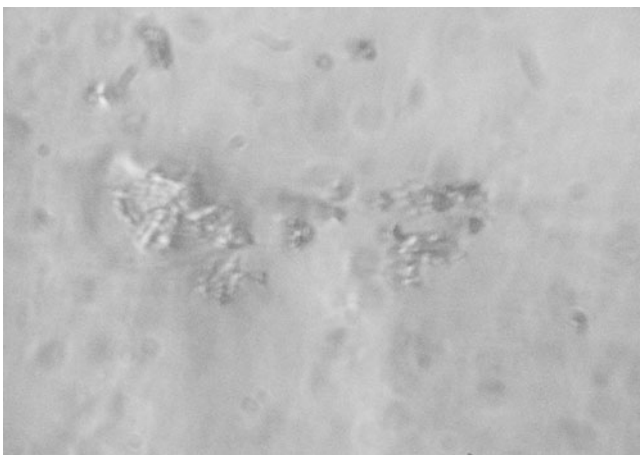

Fig. 2 Photomicrographs of destroyed *HeLa* cells (stained by AMD) after irradiation. Photo-destruction of cells was carried out by using the 450-Wt xenon lamp, supplied by a liquid heat filter (5% solution of copper sulfate)

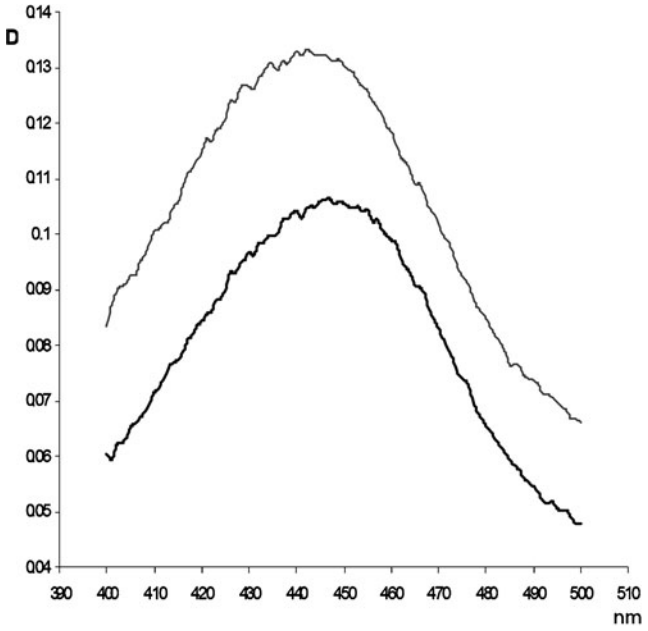

Fig. 3 Releasing AMD from *HeLa* cells to external solution after photo-destruction of cells. The below curve is the absorption spectrum of AMD in cells. The above curve is the spectrum after irradiation

the exciting light or (and) it is strongly quenched by something in the cells. However, after long incubation, when the quantity of 7AAMD in cells becomes enough much, it starts to fluoresce very intensely—almost an order of magnitude stronger than initially in solution.

When the polarized measurement of the 7AAMD fluorescence in the *HeLa* cells (after incubation with the antibiotic) was done, the fairly high value of polarization degree P=0.33 was obtained. Since the 7AAMD lifetime in *HeLa* cells is ~1.5 ns (τ in water is less than 0.9 ns, and in complex with DNA—about of 2 ns) and the volume of antibiotic molecules is approximately 400 Å^3, thus, the micro-viscosity η around of 7AAMD molecules can be found by the known Levshin-Perrin equation [5]. The calculation gives that η is about 2.6 poise. Therefore, the antibiotic inside cells places in rigidly viscous structures, but not in water phase of cytoplasm. Inside *HeLa* cells, the molecules of 7AAMD have to be bound with nuclear and mitochondrial DNA and also with oligonucleotides.

Besides, it was found, that the antibiotic interacts also with hydrophobic proteins. When 7AAMD or AMD penetrates into cells, the quenching of tryptophan fluorescence of proteins was recorded. Tryptophan is the most hydrophobic residue of proteins. Data for quenching of tryptophan fluorescence by AMD are shown in Table 2. To exclude the external shielding of the tryptophan absorption band by molecules of the antibiotic in solution, the measurements were carried out after incubation of *HeLa* cells with antibiotic for 30 min with subsequent re-precipitation of the cells, accumulated the antibiotic inside.

It is interesting that AMD in combination with caffeine quenches the tryptophan fluorescence of proteins in cells less actively than without caffeine. A possible reason is that AMD, adsorbed on the surface of caffeine clusters, is hardly pass to cellular proteins.

Using the optical microscopy for visualization of the antibiotic in cells and digital photography with camera, it was shown that AMD and its complex with caffeine at 20 °C were adsorbed on the plasma membrane of *HeLa* cells during first 10 min and only then a slow penetration into cells during 30-min incubation was observed (data are not shown in figures).

Photo-Destruction of Cells

It was found that AMD significantly contributes to photo-destruction of cells. After incorporation of AMD in *HeLa* cells, about 60% of cells were totally destroyed by short-time (3–5 min) light irradiation from 450-Wt xenon lamp (with heat filter—copper sulfate solution). In control samples, without antibiotic, the light irradiation led to only 20% destruction of cells. The photo (Fig. 1) shows an example for two *HeLa* cells, stained by AMD. After the light irradiation switch on, both cells are completely destroyed (Fig. 2) during 3 min. In suspension, the most part (up to 90%) of cell population was strongly damaged.

When photo-destruction of *HeLa* cells in suspension occurs, the antibiotic is released out from them, and the optical density of the obtained solution sufficiently increases (Fig. 3). This clearly indicates that the light absorption ability of antibiotic in cells is substantially lower than in solution.

Photo-destruction of cells leads to releasing antibiotic to an external water phase. This process is accompanied by a short-wavelength shift (~ 5 nm) of the absorption maximum of AMD. Apparently, the spectrum, recorded after photo-destruction of *HeLa* cells, is shifted due to releasing AMD from DNA and proteins of the destroyed cell to water phase.

The obtained data agree with the known fact that binding AMD with DNA in aqueous solution leads to decrease in the intensity of it absorption band and to long-wavelength shift of the spectrum by 5–10 nm [12, 13].

Conclusion

Spectroscopic and microscopic studies on application of actinomycins as anti-tumor photo-sensitizing drugs were carried out. Inside cells, 7AAMD and AMD bind not only with DNA, but also with hydrophobic proteins. Fluorescence of 7AAMD in *HeLa* cells and photo-destruction of

these cells by AMD were detected. When photo-destruction occurs, the antibiotic is released out from destroyed cells.

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