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Antiviral Research 58 (2003) 131–137

Antiviral Research

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Direct antiviral effect of cycloferon (10-carboxymethyl-9-acridanone) against adenovirus type 6 in vitro

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Received 20 September 2002; accepted 31 October 2002

Abstract

Adenoviruses represent a broad group of human pathogens that currently have no specific and safe drugs for treatment. We demonstrated direct (non IFN-mediated) antiviral activity of cycloferon (10-carboxymethyl-9-acridanone, CMA), a potent interferon inducer, against adenovirus type 6 (Ad6) in Hep-2 cells. Virus production and details of morphogenesis were studied by ELISA with antibodies to the Ad6 hexon protein, and transmission electron microscopy, respectively. Immunoenzyme assay revealed that CMA does not inhibit viral protein synthesis but instead strongly reduces the ability of the virus to generate infectious progeny virus in a dose dependent manner. Ultrastructural study shows that CMA alters the structure of intranuclear virus-specific inclusions. We suggest that CMA suppresses the late stages of viral cycle in the infected cell.

Keywords: Acridone; Adenovirus; Antiviral; Electron microscopy

1. Introduction

Due to the wide range of their tissue tropisms, adenoviruses (AdVs) cause numerous diseases such as respiratory infections, keratoconjunctivitis, cryptic enteric infection and gastroenteritis (Mei and Wadell, 1995). They are also one of the reasons for post-transplantational infections, like hemorrhagic cystitis, enteritis and hepatitis (Hoffman et al., 2001).

The AdV particle is icosahedral in shape and contains linear double-strand DNA. The protein moiety includes 11–15 structural proteins. The adenoviral cycle of reproduction in infected cells has several stages. During virus absorption to cells, viral surface proteins interact with the attachment receptors (reviewed in Schneider-Schaulies, 2000; Nemerow and Stewart, 1999), thus, enabling viral invasion into cells by endocytosis. After viral DNA is transported into the nucleus, viral genes are transcribed and translated, resulting in synthesis of structural and non-structural protein products. Expression of immediate early viral genes is followed by the first cycle of viral DNA replication, while replication of cellular DNA and protein synthesis are almost fully repressed. Morphologically, these processes lead to the formation of intranuclear virus-specific inclusions of several types containing different viral components and representing different stages of the viral cycle (Puvion-Dutilleul et al., 1998, and references therein; Lutz et al., 1996).

Simultaneously, the viral DNA packaging into newly formed capsids occurs. Hexon, penton, and fiber proteins, as well as other structural and scaffolding proteins, form pre-capsids in which viral DNA is encapsulated, leading to the formation of mature virions through several intermediate stages (reviewed in D'Halluin, 1995). Hexon is a trimeric virus-specific protein that is a major part of structural components of virion. It represents approximately 60% of virion mass and plays a leading role in immune reactions during adenoviral infection. In course of viral replication, the hexon protein is synthesized in the host cell in amounts much exceeding those needed for virus particles formation: only 20–30% of it is included into virions. The rest forms a soluble pool of the protein (Ginsberg, 1979).

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0166-3542/02/$ – see front matter
PII: S0166-3542(02)00193-6

Currently, there are no effective drugs to treat AdV infection. Several compounds of dissimilar chemical structures were evaluated prospectively for their effectiveness in the virus-infected patients with conjunctivitis or with complications after bone marrow transplantation. The results appeared to be inconclusive. For example, cidofovir (*S*-1-(3-hydroxy-2-phosphonylmetoxy propyl) cytosine, HPMPC), which is currently used to treat CMV infection, has been tried. One study demonstrated that cidofovir suppresses AdV infection effectively without cytotoxicity (Hoffman et al., 2001). However, concurrently AdV strains resistant to HPMPC were reported (Romanowski et al., 2001, and references therein). Furthermore, Kaneko et al. (2000) compared the relative effectiveness of cidofovir and another antiviral, ddC (zalcitabine), which is used for treatment of AIDS patients, to treat AdV infection. Cidofovir was reported to be less effective than ddC. Clinical usefulness of antivirals like ribavirin—now in use to treat respiratory syncitial virus infection, and gancyclovir—effective against human cytomegalovirus infection, was also evaluated to treat AdV infection. The results are contradictory with ribavirin: some studies found it to be effective (Arav-Boger et al., 2000; Shetty et al., 2000) while others did not (Bordigoni et al., 2001; La Rosa et al., 2001). Gancyclovir was reported to be active both in vitro and in vivo to treat AdV infection (Trousdale et al., 1994) and to treat AdV-associated cystitis (Chen et al., 1997).

Studies with other compounds like lipids (Kaneko et al., 2001), imidazoquinolinamines (Rautenschlein et al., 2000) and nucleoside analogs (Das et al., 1999; Gordon et al., 1991) were reported to show some antiviral activity.

Due to certain biochemical features, AdVs are highly resistant to interferon (IFN). Their replication does not depend on the presence of IFNα. It is resistant to IFNβ, and is either sensitive (Mistchenko et al., 1987; Mistchenko and Falcoff, 1987) or resistant (Kajon and Spindler, 2000; Leonard and Sen, 1996) to IFNγ. In the case of sensitivity, IFNγ does not alter absorption of AdVs or penetration of virions into cells but it does suppress the de novo synthesis of their structural components—hexon protein, PIII, PIV and PV. AdV resistance to IFN depends on low-molecular weight viral transcript VAI (for virus-associated RNA type I) which represses IFN-induced kinase, thus, interrupting the signal transduction for IFN-mediated antiviral response (Zhang and Schneider, 1993).

Cycloferon (10-carboxymethyl-9-acridanone, CMA) is a synthetic derivative of acridone and is close in chemical structure to some alkaloids from *Citrus* plants (Takemura et al., 1995; Yamamoto et al., 1989). The main feature that substantiates its clinical application is the ability to induce production of IFN at high titers (Storch et al., 1986). Now it is being used in clinical practice for treatment of various pathologies, such as influenza, herpesvirus infection, multiple sclerosis and others (reviewed by Tazulakhova et al., 2001). Here we describe the direct (non IFN-mediated) antiviral effect of the potent inducer of IFNα/β-acridone derivative CMA to AdV type 6 (Ad6) in Hep-2 cells.

2. Materials and methods

2.1. Cells and virus

Ad6 was obtained from the Influenza Research Institute viral collection and adapted for Hep-2 cells by several passages in this cell line. Hep-2 cells were grown and maintained in Eagle's minimum essential medium (MEM) with 2% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin in 96-well microplates (Sarstedt, Germany).

2.2. Determination of toxicity of CMA

To determine the cytotoxicity, Hep-2 cells were incubated with serial (100, 250, 500, 750, 1000 and 1250 μg/ml in the growth medium) dilutions of the drug and incubated at 37 °C. After 72 h of incubation the integrity of the cell monolayers was assessed visually by determining the degree of cell destruction and expressed as follows: (−), absence of any destruction (intact cells); (+), destruction up to 25%; (++), 25–50%; (+++), 50–75%; (++++), 75–100% of the cells. Based on these data, the medium cytotoxic dose of CMA (CTD_{50}) was calculated that caused death of 50% of cells.

2.3. Immunoenzyme assay (ELISA)

After the appearance of cytopathogenic effect (CPE) in control wells (infected by virus and without compounds), cells were washed three times with phosphate-buffered saline (PBS), pH 7.6, fixed in 80% cold acetone for 10 min at −10 °C, and washed for 5 min with distilled water. Monoclonal antibodies against Ad6, hexon protein were diluted to 5 μg/ml in 5% fat-free milk in PBS and incubated with fixed cells for 1 h at 37 °C. After washing three times with PBS, cells were incubated with diluted (1:10,000) goat antimouse horseradish peroxidase-conjugated antibodies (Sigma Chemical Co., St. Louis, MO) for 1 h at 37 °C. Unbound antibodies were removed by washing three times in PBS, and color reaction was developed by adding 3,3′,5,5′-tetramethylbenzidine and 0.03% H_2O_2 in 0.1 M acetate buffer, pH 5.0. The reaction was stopped with 2 N H_2SO_4, and the optical density of wells was measured at a wavelength of 450 nm. The ELISA reaction was considered positive if optical density (OD_{450}) exceeded the value of corresponding uninfected control cells by more than two times.

2.4. Virus titration

Hep-2 monolayers were prepared in 96-well microplates. Cells were inoculated with 50 μl of virus' decimal dilutions.

Table 1
Effect of CMA on Ad6 replication in Hep-2 cells

Antiviral	1000 OD_{450} at m.o.i., MID		Infectious titer of viral progeny at m.o.i., MID	
	10	100	10	100
CMA (150 μg/ml)	223 ± 14.1	359 ± 15.2	1:200	1:1,600
CMA (50 μg/ml)	244 ± 14.2	346 ± 9.4	1:200	1:3,200
IFNα (200 units/ml)	285 ± 16.4	397 ± 13.8	1:800	1:12,800
Control[a] (virus without compounds)	259 ± 15.9	361 ± 22.5	1:1,600	1:25,600

[a] OD_{450} for uninfected cells did not exceed 0.05.

The final dilution that caused positive ELISA reaction at 7 days after infection was considered a minimal infecting dose (MID).

To determine the effects of CMA and IFNα on AdV replication and infectivity of the progeny virus, cells were infected with Ad6 at multiplicity of infection (m.o.i.) of 10 or 10^2 MID, incubated with CMA (50 or 150 μg/ml), or 200 units/ml of recombinant human IFNα for 1 h at 37 °C, washed twice with Eagle's MEM to remove unbound virions, and incubated at 37 °C. The medium in the wells was changed every 24 h to eliminate effect of degradation of substances.

Infectivity of the progeny virus was determined by the ELISA assay. Infectivity was expressed as the reciprocal of the final dilution of the virus that generated a positive ELISA response. To determine the titer, serial dilutions were prepared from the virus stocks from corresponding wells (1:100, 1:200, 1:400, see Table 1), and used to infect Hep-2 cells. The final dilution that generated a positive ELISA response was considered as a virus titer.

2.5. Transmission electron microscopy (EM)

After 12 or 24 h of incubation, cells were collected from the wells, transferred into tubes, and centrifuged at 2000 × *g* for 15 min. Cell pellets were fixed with 1.5% glutaraldehyde in PBS overnight, followed by post-fixation with 1.5% OsO_4 for 1 h and uranyl acetate for 45 min at room temperature. They were then dehydrated in graded acetone and embedded in Epon/Araldit resin (Serva Feinbiochemica, Heidelberg, Germany). Thin sections (90 nm) were stained with lead citrate and examined in a JEM-100S electron microscope (JEOL, Tokyo, Japan) at 2000–50,000 instrumental magnification.

3. Results

3.1. Cytotoxicity of CMA

As was estimated after study of CMA cytotoxicity, Hep-2 cells remained intact at CMA concentrations up to 750 μg/ml, while 100% of monolayer destruction was achieved at 1000 and 1250 μg/ml. Based on these data, CTD_{50} for CMA in Hep-2 cells was estimated approximately 875 μg/ml that corresponds to 3.3 mM of compound.

3.2. Effect of CMA on viral replication and infectivity

Cells were fixed and analyzed by ELISA at Day 4 after infection when clear signs of virus-specific CPE have appeared. Results of study of the CMA effect on viral replication and infectivity are summarized in Table 1. Experiment was repeated three times, each variant of virus/substance combination has been performed in nine parallel wells of the plate, and means ± S.D. are represented in the table. Neither CMA nor IFNα treatment led to a statistically significant decrease of Ad6 hexon protein production, at the concentrations used. Nevertheless, the viral progeny were much less infectious in the presence of CMA in a dose-dependent manner. At CMA concentration 150 μg/ml, its titer was decreased eight-fold at m.o.i. of 100 MID, and 16-fold at m.o.i. of 10 MID comparing to the control. At CMA concentration 50 μg/ml, it was decreased eight-fold both at m.o.i. of 100 and 10 MID.

3.3. Effect of CMA on Ad6 morphogenesis

The experiments have been performed three times and the resulting EM pictures are representative for numerous cells studied in all three experiments.

As ultrastructural study 12 h post infection (p.i.) revealed, morphologically distinguishable inclusions of three types appeared in cell nuclei (Fig. 1):

1. Small, spherical electron-dense inclusions (type I);
2. Extensive inclusions of irregular shape and high electron density (type II);
3. Extensive diffuse inclusions of medium or low electron density (type III).

Twenty-four hour p.i., mature virions appeared in cell nuclei. They were located along the type II and III inclusions and had an electron-dense core or ring-like structures at their centers. Inclusions of types II and III at this time formed large conglomerates occupying most of the nuclear space. In some cases, crystal formations of highly ordered fibrillar structure (type IV inclusions) were observed. Mature virions formed paracrystalline structures (type V inclusions, Fig. 2),

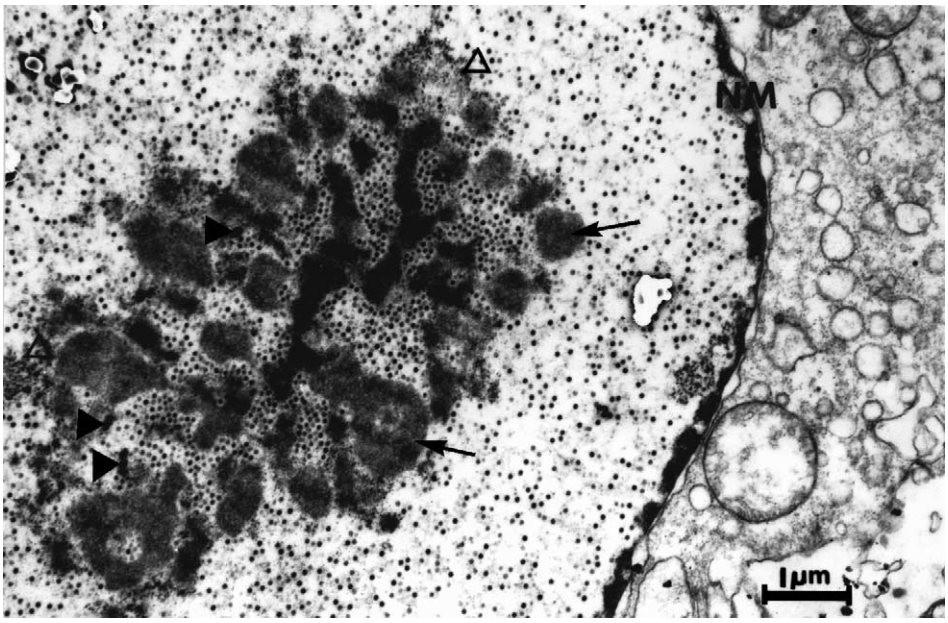

Fig. 1. Adenoviral inclusions type I (arrowheads), type II (arrows), type III (empty arrowheads) and mature virions in the nucleus of a Hep-2 cell 24 h p.i. Magnification: 4000×; bar, 1 μm. NM, nuclear membrane.

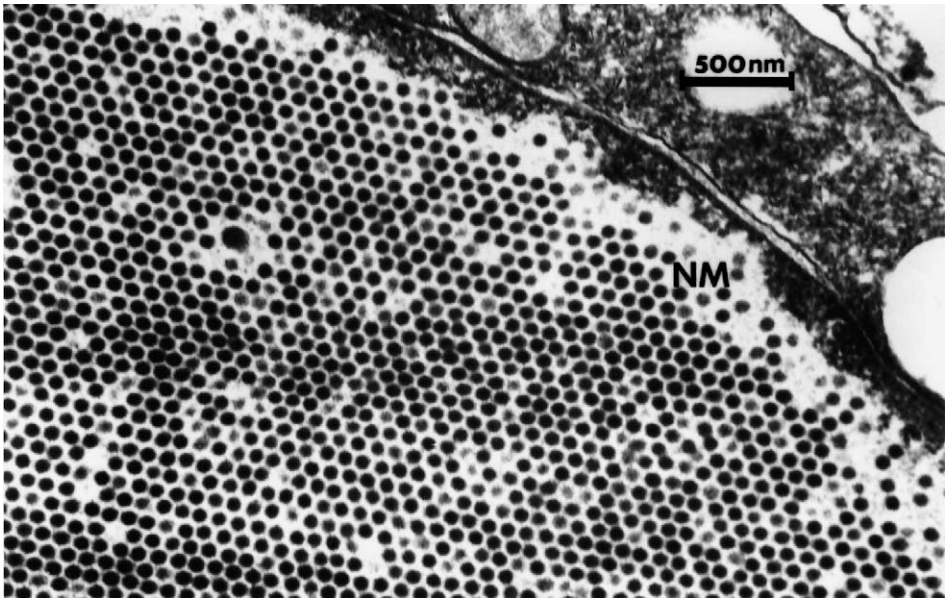

Fig. 2. Adenoviral intranuclear inclusion formed by mature virions after 24 h p.i. Magnification: 15,000×; bar, 500 nm. NM, nuclear membrane.

which were transported into the cytoplasm and the extracellular space.

In the presence of CMA, significant alteration in viral morphogenesis was observed. Twenty-four hour p.i., in cell nuclei, virions were not produced at all, despite the presence of highly developed virus-specific inclusions of types II and III. Type II inclusions themselves were more porous and less electron-dense in appearance (Fig. 3). In some cases, newly assembled virions and their conglomerates were not attached to nuclear sites of viral replication. This appearance has only rarely been observed in control cultures.

4. Discussion

We have demonstrated the direct antiadenoviral effect of CMA that had not been mediated by IFN. Previously (Storch et al., 1986), CMA has been found to be a highly potent inducer of IFNα/β but not IFNγ, thus, making it a convenient substance for adenoviral research, due to the resistance of AdVs to the IFNα/β.

It is concluded from the results of the ELISA analysis, the presence of CMA does not inhibit the production of Ad6 hexon protein, but it does strongly reduce the yield

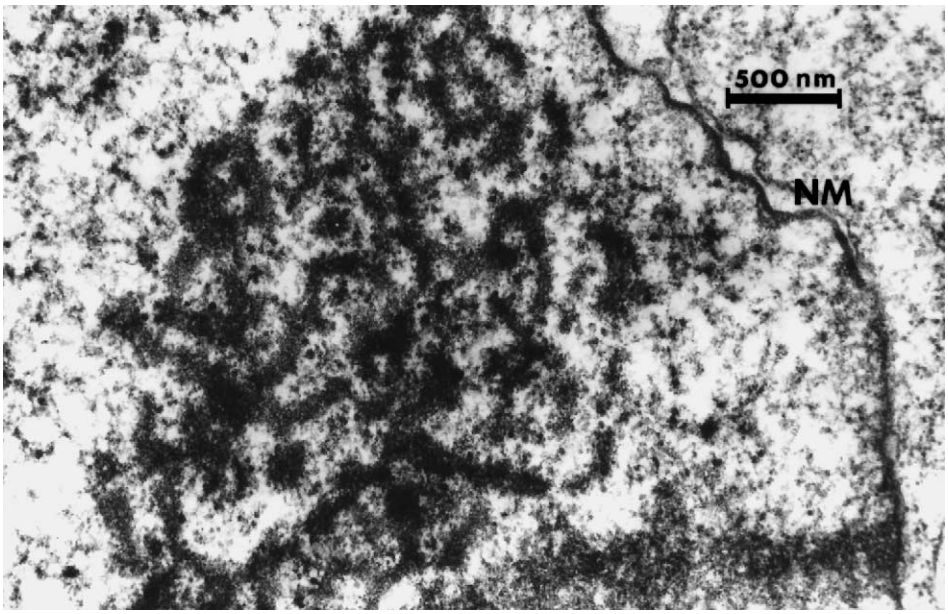

Fig. 3. Adenoviral type II inclusion in the nucleus of Hep-2 cell 24 h p.i. in the presence of CMA. No mature virions can be detected. Magnification: 15,000×; bar, 500 nm. NM, nuclear membrane.

of infectious virus. We, therefore, propose that the target point of CMA action is not the primary viral transcription or translation, but rather late DNA replication and/or packaging into virions during viral assembly. This hypothesis has been partially supported by the results of ultrastructural examination of infected cells, namely, the altered structure of virus-specific inclusions.

In general, the ultrastructural picture of adenoviral infection is in good agreement with previous results (Puvion-Dutilleul et al., 1998, and references therein; Murti et al., 1990), allowing us to identify the type III and II intranuclear inclusions as sites of DNA replication and viral genome storage, respectively.

Chaly and Chen (1993) and Puvion-Dutilleul et al. (1998) presented detailed electron microscopic studies of type 5 AdV replication in HeLa cells. Our results differ slightly from theirs, a fact which could be explained by both different virus strain and different cell line. In particular, we did not distinguish as many types of viral intranuclear inclusions, but only five of them. Nevertheless, inclusions of two main types (II and III) were clearly distinguishable, and the changes to them, as a result of CMA presence, were obvious.

Acridone derivatives are currently under intense study as potential anticancer and antiviral agents. They have been demonstrated to inhibit tumor cell proliferation (Tabarrini et al., 1999; Gamage et al., 1992), and their antiherpesvirus effect has been shown as well (Akanitapichat et al., 2000). Carboxymethyl derivatives of acridone substituted in position 4 have been shown to reduce colon tumor development by activation of necrosis (Gamage et al., 1992). Several different cellular metabolic points have been suggested to be molecular targets for their effect, such as the PKC-dependent mechanism during HIV activation from its latent stage (Turpin et al., 1998), topoisomerase II (Akanitapichat et al., 2000; Bastow et al., 1994), transmembrane pump protein providing multidrug resistance of cancer cell lines (Evers et al., 2000) and mitochondrial enzyme NADH: ubiquinone oxidoreductase (Oettmeier et al., 1995, and references therein). Although we cannot exclude the potential role of all these points, as well as another still unknown, in altering the Ad6 reproduction in cells by CMA, the topoisomerase inhibition by the substance appears to be the most important in this process. This speculation is based on results obtained by some authors (Schaak et al., 1990; Wong and Hsu, 1990), who showed that repression of topoisomerase I decreases the transcription of AdV early genes and viral DNA replication in the late stages of infection, while topoisomerase II inhibitor represses the enzyme-directed ligation of non-replicating viral DNA and its packaging into capsids. The importance of topoisomerases for AdV replication was indirectly confirmed by a more than 10-fold increase in topoisomerase gene transcription during the course of infection (Chow and Pearson, 1985), although some authors found no increase in the amount of topoisomerase protein or enzyme activity (Romig and Richter, 1990). Type II AdV-specific inclusions are considered to be viral genome storage sites in the cell nucleus (Puvion-Dutilleul and Pichard, 1992). The fact that CMA alters their structure may serve as strong support of this hypothesis of CMA's mechanism of antiviral action. Previously we demonstrated the accumulation of CMA in cell nuclei rather than in the cytoplasm, supporting the idea that the nucleus is the main site of CMA activity (Kovalenko et al., 2000). It appears logical that the virus, with such a strong ability to activate nuclear metabolism and to reorganize its structure for viral DNA replication, needs increasing amounts of topoisomerase and is sensitive

to its absence. The structure and mode of action of some topoisomerase poisons, including one acridone derivative (mitoxantrone) are reviewed by Froelich-Ammon and Osheroff (1995). Recently (Akanitapichat and Bastow, 2002), acridone derivative was demonstrated to interfere with processes of herpesviral DNA maturation. Due to inability of the virus to develop a resistance to the compound, authors have suggested it to be prospective candidate for further development as an effective therapeutic agent.

Another possible way of virus-inhibiting action of CMA could be based on its ability to overcome VAI-induced repression of IFN synthesis that is typical for adenoviral infection (Zhang and Schneider, 1993). Of course, the exact mechanism of antiviral action of CMA should be a subject for further investigations.

These results, taken together with the low toxicity of CMA and its strong IFN-inducing ability (Romer et al., 1986; Storch et al., 1986), suggest that it should be considered further as a multi-factorial and prospective antiviral substance.

Acknowledgements

We thank Fogarty International Center for partial support of the study. We also thank Kathy Cavanagh from Wadsworth Center, New York Department of Health for the critical review and correction of the manuscript. This work was supported in part by grants #1D43TW0091 and 1D43TW002 from the Fogarty International Center/NIH.

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