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Small Molecule Therapeutics

Molecular Cancer Therapeutics

Oncolytic Adenovirus Loaded with L-carnosine as Novel Strategy to Enhance the Antitumor Activity

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Abstract

Oncolytic viruses are able to specifically replicate, infect, and kill only cancer cells. Their combination with chemotherapeutic drugs has shown promising results due to the synergistic action of virus and drugs; the combinatorial therapy is considered a potential clinically relevant approach for cancer. In this study, we optimized a strategy to absorb peptides on the viral capsid, based on electrostatic interaction, and used this strategy to deliver an active antitumor drug. We used L-carnosine, a naturally occurring histidine dipeptide with a significant antiproliferative activity. An *ad hoc* modified, positively charged L-carnosine was combined with the capsid of an oncolytic adenovirus to generate an electrostatic virus–carnosine complex. This complex showed enhanced antitumor efficacy *in vitro* and *in vivo* in different tumor models. In HCT-116 colorectal and A549 lung cancer cell lines, the complex showed higher transduction ratio and infectious titer compared with an uncoated oncolytic adenovirus. The *in vivo* efficacy of the complex was tested in lung and colon cancer xenograft models, showing a significant reduction in tumor growth. Importantly, we investigated the molecular mechanisms underlying the effects of complex on tumor growth reduction. We found that complex induces apoptosis in both cell lines, by using two different mechanisms, enhancing viral replication and affecting the expression of Hsp27. Our system could be used in future studies also for delivery of other bioactive drugs. *Mol Cancer Ther; 15(4); 1–10. ©2016 AACR.*

Introduction

Oncolytic adenoviruses are genetically modified adenoviruses able to kill cells through the expression of cytotoxic proteins and cell lysis (1). They are used for the treatment of cancer because of their ability to replicate selectively in tumor cells (2).

It has been showed that oncolytic adenovirus, used as a single agent, cannot fully eradicate tumors. Therefore, current strategies involve the combination of oncolytic adenoviruses with chemotherapy, radiation, or immunotherapy.

During the recent years, considerable attention has been given to the use of natural substances as anticancer drugs. The natural antioxidant dipeptide L-carnosine belongs to this class of molecules because it has been proven to have a significant anticancer activity both *in vitro* and *in vivo* (3). A number of biologic functions have been recognized to this molecule, including antioxidant activity, ability to chelate metal ions, inhibition of protein glycosylation, anti-inflammatory, and antisenescence properties (4). However, one of the limitations of the use of L-carnosine is that the dose needed to arrest tumor progression exceeds the one currently used in clinical settings (5, 6). The aim of this study was to overcome this limitation by loading the carnosine on the surface of an oncolytic virus to facilitate the entry of the peptide into cancer cells. Consistent with this hypothesis, we found that a Carnosine6K-coated oncolytic adenovirus displays an increased transduction efficacy and enhanced infectious titer compared with the uncoated oncolytic adenovirus. In addition, both *in vitro* and *in vivo*, we demonstrated that oncolytic virus complexed with Carnosine6K showed an evident synergistic cytotoxic effect. These results encourage the use of oncolytic adenoviruses coated with modified carnosine for new therapeutic protocols to treat human colon cancer and lung cancer. Moreover, this strategy could be exploited for the delivery of many other bioactive drugs.

Materials and Methods

Cell lines

HCT-116 cell lines were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and obtained from the cell bank of CEINGE Biotechnology in

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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2014. They were constantly monitored in our laboratory by microscopy morphology check and mycoplasma presence. A549 lung cancer cell lines were purchased from the ATCC (American Type Culture Collection) in 2012, and they were checked for mycoplasma and sterility before the use and routinely monitored in our laboratory by microscopic morphology check. CCD-112Sk, human skin fibroblasts were kindly provided by Dr. Santos from University of Helsinki (Helsinki, Finland) and no authentication was done by the authors. Cells were cultured at 37°C and 5% CO_2 in DMEM purchased from BioWhittaker Classic Cell Culture Media, Lonza - VWR International s.r.l., supplemented with 10% FBS (Gibco Laboratories) and 1% penicillin/streptomycin (Gibco Laboratories).

Virus preparation

The oncolytic adenoviruses utilized in this work were all prepared according to standard protocols (7). Ad5D24-Rfp was kindly provided by Dr. Masataka Suzuki from Baylor College of Medicine (Houston, TX; ref.8) and it was propagated in our laboratory.

Treatments

L-carnosine was purchased from Sigma-Aldrich, and dissolved in PBS (Lonza-VWR International s.r.l.). A modified version of L-carnosine (Carnosine6K), featuring six additional lysines at the C-terminus, was purchased from GeneCust Europe Laboratoire de Biotechnologie du Luxembourg S.A, and dissolved in PBS (Lonza-VWR International s.r.l).

Complex formation

To saturate the surface of the oncolytic adenovirus, an excess of Carnosine6K was used. Previous studies performed in our laboratory showed that a ratio of 1:500 virus-to-peptide protein amount was sufficient to efficiently coat the viral surface. To allow the formation of the complex between the oncolytic virus and Carnosine6K, the two components were mixed and incubated at room temperature for 15 minutes using MilliQ H_2O at pH 7.4 as buffer (9).

Zeta potential and dynamic light scattering analysis

Zeta potential analysis has been performed using 1×10^{10} viral particles (VP). All the samples were diluted in a volume of 800 μL of MilliQ H_20 at pH 7.4 and injected with a 1 mL syringe in the capillary flow cell to measure the electric surface charge of the particles. An equilibration time of 120 seconds was set on the software to allow the samples to stabilize at 25°C. For the Dynamic light scattering analysis, the measurements were performed with a ZetaSizer Nano Malvern.

Viability and transduction assay

Cell viability was determined by MTS according to the manufacturer's protocol (Cell Titer 96 AQueous One Solution Cell Proliferation Assay; Promega). Cells were seeded at a density of 1×10^4 cells/well in 96-well plates and maintained under appropriate condition. On the following day, cells were infected using an oncolytic adenovirus Ad5D24Rfp encoding for the red fluorescent protein (100 VP, 10 VP, 1 VP, 0.1 VP) and complex (100 VP, 10 VP, 1 VP, 0.1 VP). Red fluorescence was measured by Varioskan plate reader at 24, 48, and 72 hours after the treatment.

Determination of the infectious titer

The determination of the infectivity was based on the visual quantification of infected cells. Upon the infection, the cell synthesized excessive amounts of hexon proteins, which were targeted by the first antibody and diluted 1:2,000 for 1 hour at room temperature in the dark (NB600-413, Novus Biologicals). Then the cells were washed and incubated with the second Biotin-SP–conjugated antibody and diluted 1:500 for 1 hour at room temperature in the dark (115-065-062, Jackson Immuno Research). After the incubation time, cells were washed and incubated in the dark with Extravidin-peroxidase for 30 minutes at room temperature (E2886-1ML, Sigma-Aldrich). Finally, cells were treated with DAB (D3939-1SET, Sigma-Aldrich). The detection of infectious titer was made using microscope EVOS, and each well was photographed (5 pcs) at five nonoverlapping sites. Infected cells were counted using ImageJ and the infectious titer was calculated according to the following equation:

$$\text{Infectious titer: } x\frac{A(\text{well})11\text{mL}}{A(\textit{field})1V}$$

Where x = number of infected (stained cells)
A (24 well) = 190 mm^2
A (field) = surface area of the field
L = dilution
V = volume of virus dilution applied per well

Analysis of apoptotic and necrotic cell death

The amount of apoptotic and necrotic cells were measured after 24 and 48 hours postinfection with a TACS Annexin V-FITC kit (Trevigen Inc.) and BD LSR II flow cytometer according to manufacturer's instructions.

Western blot analysis

Total extracts of HCT-116 and A549 cell lines were probed with antibodies against p62 (H00008878-M01; mouse mAb from Abnova), LC-3 (#2775, rabbit polyclonal antibody from Cell Signaling Technology, Inc), HSP27 (ab2790, mouse mAb from Abcam), and β-actin (sc-47778, mouse mAb from Santa Cruz Biotechnology). The signals were detected using the ECL kit (K-12045-D50, Advansta).

Quantitative PCR

qPCR for adenoviral E4 was performed as described previously (10).

RNA extraction and real-time PCR

Primer sequences used in qPCR are the following: IL8 forward: 5′-AGACAGCAGAGCACACAAGC-3′; IL8 reverse: 5′-ATGGTTCCTTCCGGTGGT-3′; actin forward: 5′-CCTCACCCTGAAGTACCCCA-3′; actin reverse 5′-TCGTCCCAGTTGGTGACGAT -3′

Animal experiments

All animal experiments were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were obtained from Scanbur (Karlslunde, DK) at 5–7 weeks of age and quarantined at least for 2 weeks before the study. Health status of the mice was frequently monitored and as soon as signs of pain or distress were evident they were euthanized. For the efficacy experiment, human xenografts were established by injecting

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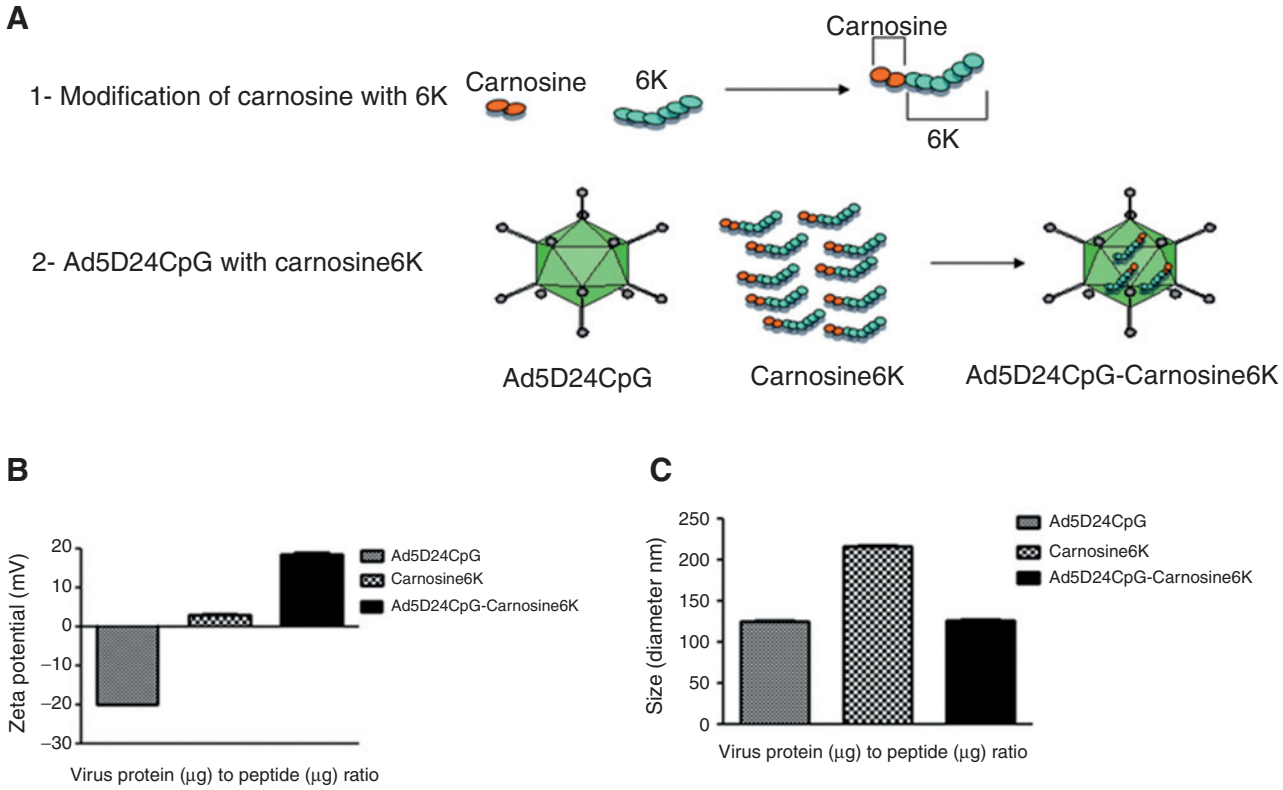

Figure 1.
Oncolytic adenovirus as a carrier for active drugs. A, schematic representing the strategy to generate the carnosine–virus complex. A poly-lysine chain was added to L-carnosine (Carnosine6K) to confer to the dipeptide a positive charge that favors the interaction with the negatively charged capsid of the virus, as L-carnosine needs to be modified to be "absorbed" on the viral capsid. B and C, 1×10^{10} viral particles were conjugated with Carnosine6K at the ratio of 1:500. After the reaction, zeta potential and particle size were measured.

1×106 A549 and HCT-116 cells s.c. into the flanks of 5–7 week-old female BALB/c nude mice ($n = 5$ per group). Tumors (two tumors per mouse, $\sim 5 \times 5$ mm in diameter) were injected i.t. with a volume of 50 μL for three times on days 0, 2, and 5 with 10^8 VP/tumor of Ad5D24CpG and control tumors were injected with PBS only. The formula (length × (width) × 0.5) was used to calculate the tumor volumes. Mice were euthanized when a tumor reached an average diameter of 15 mm.

Statistical analysis

Statistical significance was determined by unpaired, two-tailed Student *t* test using GraphPad Prism 6 (GraphPad Software, Inc.).

Therapeutic synergy was assessed using the fractional tumor volume (FTV) method (11, 12).

Results

Modified L-carnosine can be coated on the capsid of an oncolytic adenovirus through an electrostatic interaction

The main purpose of this study was to develop a new system to increase the amount of L-carnosine that is delivered to cells by using virus as a carrier. In our laboratory, we developed a system to complex MHC-I–restricted peptides with the viral capsid (9); here, this system was adapted to combine virus and L-carnosine (Fig. 1A). L-carnosine was modified by adding a poly-lysine chain (Carnosine6K) to confer to the peptide a positive charge and consequently to allow the interaction with the viral capsid that has a negative charge (13). To conduct experiments, a serotype 5 conditionally replicating adenovirus that features a 24 base pair deletion in E1A gene and enriched with CpG islands (Ad5D24CpG) to stimulate the innate immune system through toll-like receptor 9 (TLR9; ref.7) was used. It was already reported that the complex presented good stability and no significant decrease of zeta potential was observed 15, 30, and 45 minutes after incubation in the same conditions (MilliQ water ph 7.4). Moreover, no aggregation was seen at these time points (14). First, the electric surface charge (zeta potential) of the complex was analyzed to see whether the addition of the Carnosine6K was influencing the charge and the size of the adenovirus–carnosine complex (Fig. 1B). It was observed that after mixing Carnosine6K with oncolytic adenovirus (1:500), the electric surface charge became positive compared with the virus alone and Carnosine6K alone (Fig. 1B). In addition to the net charge, the diameter of the particles was also analyzed (Fig. 1C).

Carnosine6K-coated viruses display increased transduction efficacy, reduction in cell viability, and enhanced infectious titer

To ensure that viral complex did not affect healthy cells, it was tested in CCD-112Sk, human skin fibroblasts, by performing MTS cell viability assay to see whether any toxic effect was observed (Supplementary Fig. S1).

Indeed, we wanted to examine whether the presence of Carnosine6K would have an effect on the transduction efficacy of the

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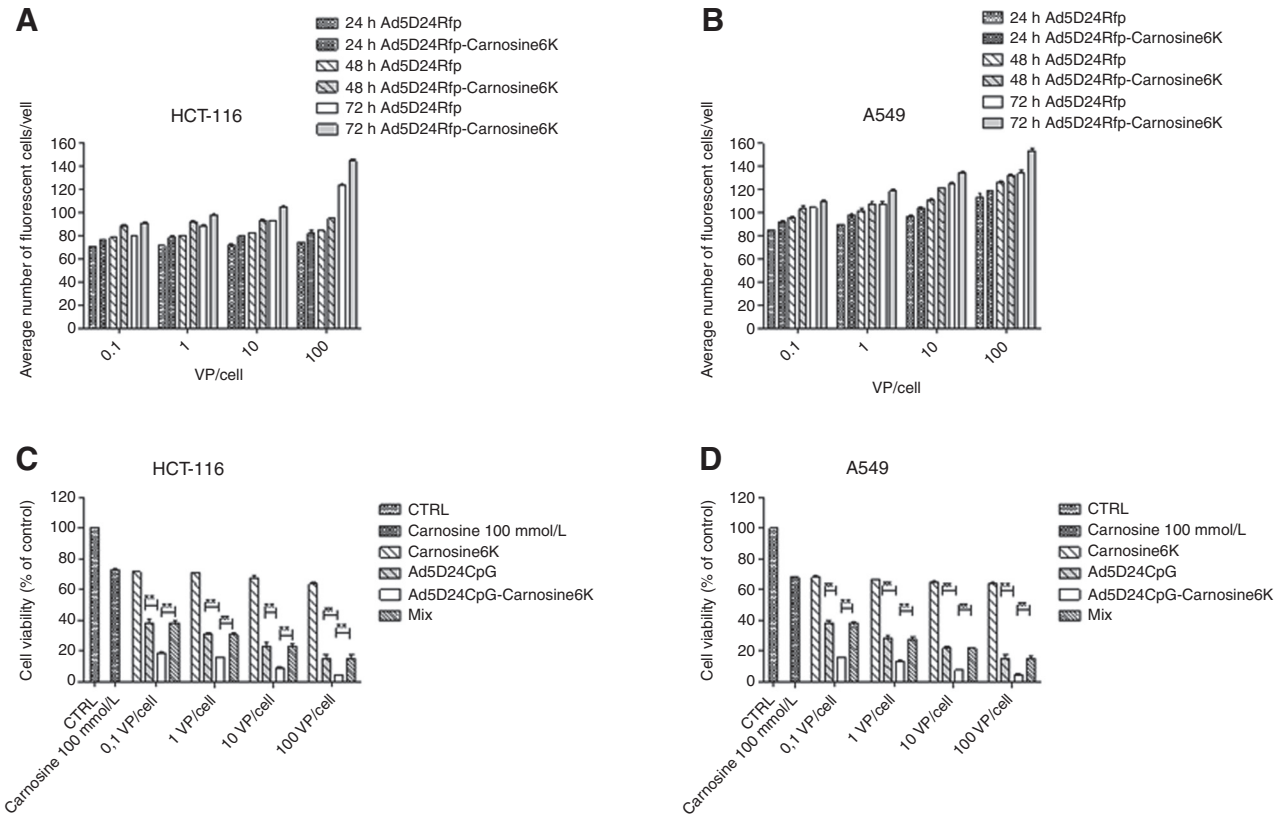

Figure 2.
Effect of Carnosine6K-coated virus on cell transduction efficacy and cell viability. A and B, the transduction efficacy was evaluated by infection with an oncolytic adenovirus encoding for the red fluorescent protein (RFP) with or without Carnosine6K. RFP was measured using Varioskan plate reader 24, 48, and 72 hours postinfection. C and D, cell viability was performed by MTS assay on two cancer cell lines. **, $P < 0.01$.

virus, we performed a transduction assay on two human tumor cell lines A549, human lung cancer cell line, and HCT-116, human colon cancer cell line. The transduction efficacy of an RFP-expressing oncolytic adenovirus (Ad5D24RFP) uncoated or complexed was compared with modified L-carnosine (Ad5D24RFP-Carnosine6K). Transduction was assessed at 24, 48, and 72 hours postinfection. Interestingly, at 72 hours from infection, the amount of fluorescent cells treated with complex was higher compared with cells treated with naked oncolytic adenovirus (Fig. 2A and B).

To assess the cell killing activity of the complex, MTS cell viability assays were performed. It was observed that at 100 VP, complex displayed enhanced activity (Fig. 2C and D; $P < 0.001$).

Then, the infectivity of the virus was determined; Ad5D24CpG–Carnosine6K complex was added as a single entity and Ad5D24CpG and Carnosine6K added separately as a mix by immunocytochemistry assay (ICC). The infectious titer was higher in the complex than in the virus alone using an infectious ratio of 10 VP/cell in both cell lines tested (Fig. 3A–D). In particular, in A549 cells, one log higher infectious titer of Ad5D24CpG–Carnosine6K complex was found compared with the uncoated.

Carnosine6K-coated viruses can induce apoptotic and necrotic cell death

Apoptosis is a physiologic response to exogenous and endogenous death signals. In particular, a prerequisite for tumor growth is the inactivation of apoptotic pathways which is also responsible for therapy resistance (15). It has been demonstrated that L-carnosine treatment at concentration 100 mmol/L is able to induce late apoptosis only in a small number of cells (6). Thus, the ability of the complex to induce apoptotic and necrotic cell death was studied. The amount of Annexin-V-positive cells, that is early apoptotic cells, and propidium iodide–positive cells (PI), that is late apoptotic/necrotic cells 24 and 48 hours after the treatment in A549 and HCT-116 (Fig 4A, B, D and E) cells was measured. The levels of fold change at 48/24 hours of both the early and the late apoptotic/necrotic cells were elevated in the complex in both cell lines, compared with the virus alone and L-carnosine alone with or without six lysines (Fig. 4C and F).

Oncolytic adenovirus loaded with L-carnosine induces autophagy and enhances virus replication

It has been reported that virus-induced autophagy correlates positively with virus replication and that the combination of an adenovirus with an autophagy inducer improves antitumor effects in cancer cells (16). L-carnosine has been defined as a mimic of rapamycin, an inhibitor of the mTOR, and so may be able to induce autophagy (17). For these reasons, autophagy and virus replication in HCT-116 and A549 cancer cell lines treated with virus, Carnosine6K, and complex was investigated.

The autophagy was analyzed by assessing the expression of LC3-I and LC3-II and p62 by immunoblotting assay. Conversion of LC3-I to LC3-II was used as a marker to estimate autophagy (18). p62 protein accumulates when autophagy is inhibited, and decreases when autophagy is induced (19). We first analyzed the

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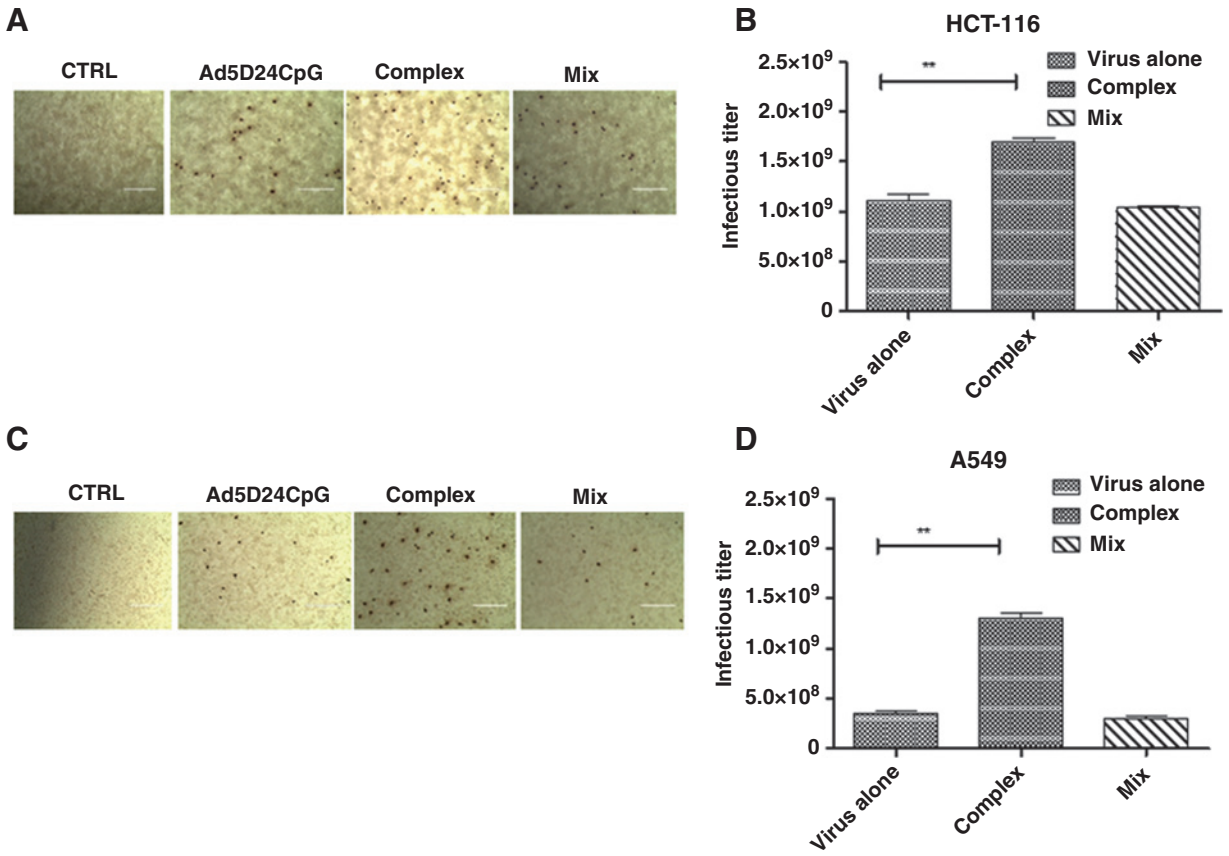

Figure 3.
Carnosine6K-coated virus shows higher infectivity in lung and colon cancer cell line. B and D, infectivity of the complex and of the naked virus was assessed by ICC assay in two different cell lines. A and C, most representative microscope photograph of the infected wells. Figures represent difference in hexon protein expression (virus assembling), without distinguishing infectivity, replication, or gene expression manner. **, $P < 0.01$.

effect of Carnosine6K at 0, 24, and 48 hours after treatment. As shown in Fig. 5A, in HCT-116 cells, the amount of LC3-II readily was increased 24 hours postaddition of Carnosine6K and was further increased 48 hours after, compared with control cells. In A549 cell lines, the Carnosine6K treatment revealed a better conversion of LC3-I to LC3-II respect to untreated control cells only 48 hours after treatment (Fig. 5A'). At the same time, the intracellular levels of p62 were decreased in both cancer cells, according to LC3-I conversion (Figs. 5A, A').

Next, the effect of the complex, adenoviruses alone, or the combination of the adenovirus and Carnosine6K, reported in the text as mix on autophagy, was investigated. Our results showed an increase in the amount of LC3-II in HCT-116 cells infected with complex, compared with adenovirus alone or mix. Despite the autophagy was quite evident in A549, significant differences were not observed in complex infection with respect to virus alone or mix. Meanwhile, the intracellular p62 level was decreased at all time-points (Fig. 5B'). To assess whether the autophagy would have an influence on virus replication, the kinetics of viral genomes by quantitative PCR was evaluated. We observed in HCT-116 an increase of virus DNA after infection with complex at 24 and 48 hours, compared with the adenovirus alone or mix (Fig. 5C). In A459 cells, no significant differences were detected in virus DNA amount (Fig. 5C'). These results demonstrated that in HCT-116 cells, Carnosine6K loaded to Ad5D24CpG improves antitumor effect by enhancing the viral replication and by inducing autophagy.

Carnosine6K-coated viruses affect Hsp27 expression, modulating IL8 mRNA levels in A549 cells

Hsp27 is a stress-activated chaperone involved in protecting cells from apoptosis in both normal and cancer cells. As many other HSPs, Hsp27 is overexpressed in many cancer cells and seems to have a role in mediating viral infection signaling (20). We evaluated the effect of L-carnosine on Hsp27 expression in HCT-116 and A549 cells treated with Carnosine6K or after infection with complex with respect to adenoviruses alone or mix. The evaluation of Hsp27 expression highlights once again a difference between two cell lines. Indeed, it was found that in HCT-116 cells, the expression level of Hsp27 did not seem to be affected by Carnosine6K treatment, although a slight increase in the expression after adenovirus infection (Fig. 6A and B) was observed. It has been reported that the expression of HSPs could enhance the replication of oncolytic adenovirus; then, our results may be in agreement with the enhanced viral DNA replication in HCT-116 cells. In A549 cells, only Carnosine6K treatment reduced the expression of Hsp27 in a time-dependent manner and this reduction was more evident after infection with complex and 48 hours after the mix infection (Fig. 6C and D). This result could explain why the complex is more efficient to induce apoptosis with respect to the adenovirus alone. Indeed, it is noted that knocking-down Hsp27 expression by siRNA resulted in more apoptotic cell death in A549 cells (21). The Hsp27 also mediates the adenoviral infection signaling by forming a signalosome with p38 and

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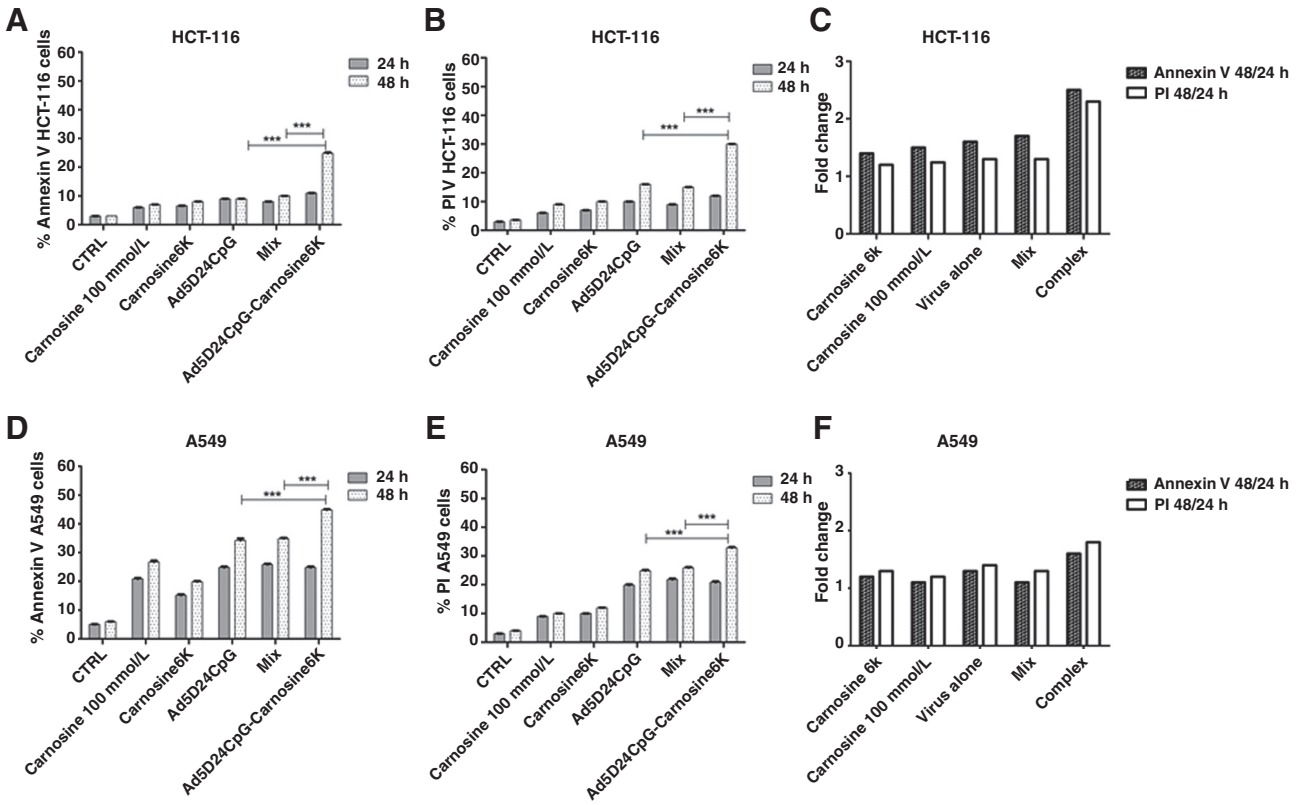

Figure 4.
Induction of apoptotic and necrotic cell death. A and B, early and late apoptotic or necrotic cell death were measured in HCT-116 cells after 24 and 48 hours postinfection. D and E, early and late apoptotic or necrotic cell death were measured in A549 cells after 24 and 48 hours postinfection. C and F, fold change over time (48/24 hours after infection). The amount of early and late apoptotic or necrotic cells 24 and 48 hours after virus infection were analyzed by flow cytometry. FITC-labeled Annexin-V was used to indicate the early apoptotic cell and PI for the necrotic or late apoptotic cells. ***, $P < 0.001$.

NfkB-p65 influencing downstream expression of proinflammatory mediators, particularly the expression of IL8, cytokine of the CXC chemokine family. IL8 contributes to tumor growth and progression, inducing cell growth and survival especially in lung cancer (20).

Then, the mRNA levels of IL8 by real-time PCR in HCT-116 and A549 were evaluated. In HCT-116 cells, the IL8 mRNA level did not change, after infection with complex or mix. On the other hand, we found a significant decrease of IL8 mRNA levels after infection with complex in A549 cells (Fig. 6E and F). The stronger antitumor effect of complex, observed in both cell lines, may be generated from the sum of the adenovirus and L-carnosine activities or even a synergistic effect (the carnosine effect may increase the efficacy of adenovirus).

Complex displays increased efficacy *in vivo* in a xenograft model of lung and colon cancer and reduces the expression of Hsp27

The oncolytic activity of the complex was tested in a lung and colon cancer xenograft model. Nude mice bearing A549 and HCT-116 cell tumors in the flanks were treated intratumorally with 1×10^8 VP/tumor (or PBS) on days 0, 2, and 5 and the tumor growth was followed over time. It was observed that the tumor growth was significantly ($P < 0.001$ at day 18) reduced in both complex-treated mice compared with the control virus and Carnosine6k alone (Fig. 7A and B). Then, the therapeutic synergy between Ad5D24CpG and Carnosine6K was calculated using fractional tumor volume (FTV) method. Synergistic effect was found in A549 mouse model treated with complex compared to mice treated with mix. Interestingly, in HCT-116 cells, a synergistic effect in complex and mix condition was found; however, the effect was stronger in mice treated with the complex (Fig. 7C and D; Supplementary Table S1).

Recent studies underline that the Hsp27 expression strongly correlates with poor survival in patients with rectal cancer (22). For this reason, the expression of Hsp27 was evaluated in protein extracts obtained from xenograft tumors. Surprisingly, it was found that the expression of Hsp27 was dramatically reduced in both xenograft tumors after intratumoral administration of the complex. (Fig. 7 E and F).

Discussion

In spite of encouraging clinical results, improvements to enhance the efficacy oncolytic adenoviruses are still needed (23, 24). One strategy is to combine the viruses with chemotherapy (25). However, the uptake of drug-like molecules might still be a problem. For this reason, the new anticancer strategies aim to deliver and facilitate the uptake and intracellular accumulation of drugs into cancer cells, while reducing the nonspecific uptake into normal cells. To overcome this limitation, the possibility to use the adenovirus as carrier for active molecules was explored (3).

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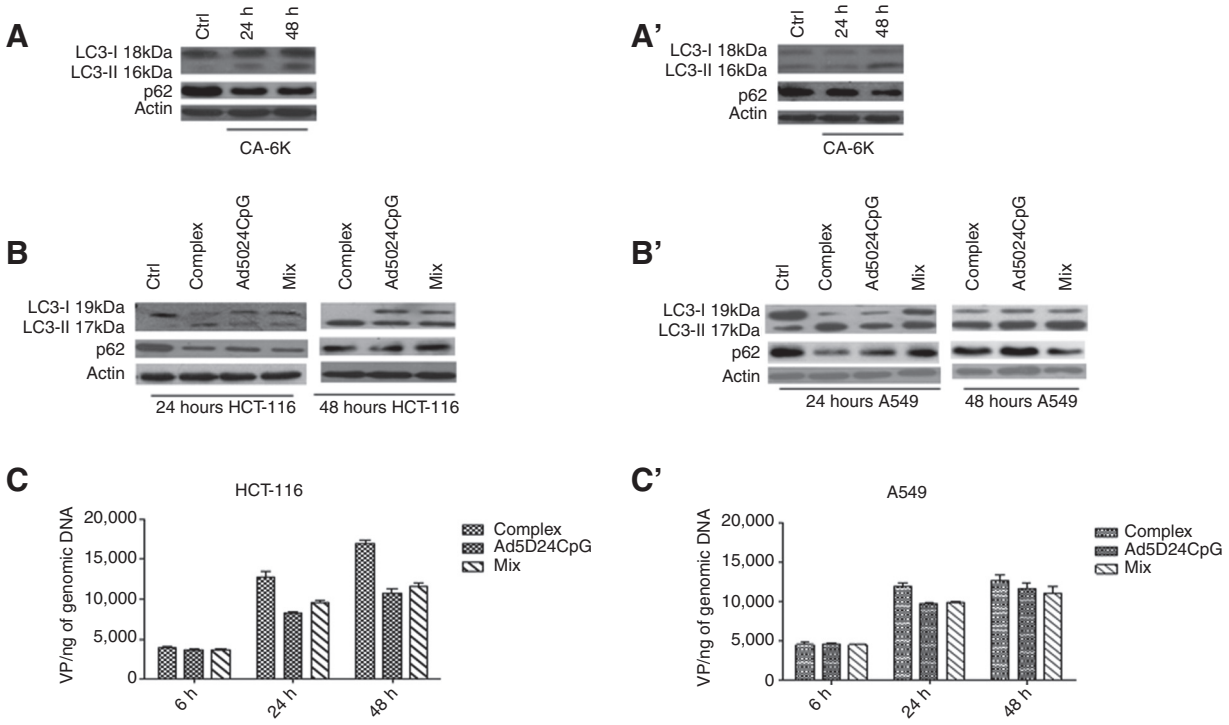

Figure 5.

Ad5D24CpG-Carnosine6K induces autophagy, enhancing virus replication. A and A′, Western blot analysis of HCT-116 and A549 cells treated with Carnosine6K (CA-6K). Cells were harvested at 0, 24, and 48 hours posttreatment and cell lysates were analyzed for expression of p62 or LC-3. β-Actin was used as a loading control. B and B′, Western blot analysis of HCT-116 and A549 cells treated with complex, Ad5D24CpG alone, or Ad5D24CpG and Carnosine6K added separately (mix). Cells were harvested at 24 and 48 hours postinfection and analyzed for expression of p62 or LC-3. β-Actin was used as a loading control. C and C′, quantification of viral DNA from HCT-116 and A549 cells, infected with Ad5D24CpG alone, complex, or mix and analyzed at 6, 24, and 48 hours postinfection by qPCR. Data shown are representative results from a total of three separate experiments. Data are presented as mean ± SD for each group.

Adenovirus capsid has been previously coated mainly with polymers to shield the virus for recognition of receptors of the innate immunity and antibodies, but our system is the first electrostatic strategy used for the delivery of active drugs by the virus (26). In accord with pervious findings (13), enhanced transduction and less receptor dependency of the positively charged complex compared with naked virus was also observed (13). Indeed, the percentage of transduction was mildly affected by the complex; however, the complex group exhibits higher amount of fluorescent cells over other groups at 72 hours post-infection. This could suggest that complex activity has been improved by Carnosine6K presence, and its mechanism of action is not directly related to the transduction as shown *in vitro* through transduction and infectivity assays and *in vivo* efficacy studies. We have previously demonstrated that L-carnosine could represent an adjuvant candidate for colon cancer treatment and the adenovirus as carrier for L-carnosine has proved to be a good strategy to improve the adenovirus activity. The role of L-carnosine in the complex was to enhance anticancer effect when combined with oncolytic adenovirus. We hypothesized that combined agents could exhibit synergistic anticancer properties, as it is known that oncolytic adenovirus are novel antitumor agents with the ability to selectively replicate and lyse tumor cells while remaining innocuous to the rest of the body (7) and L-carnosine alone has antiproliferative cancer effect (3). Indeed, we found that the complex formed by the interaction between L-carnosine and adenovirus was able to induce apoptosis and necrosis at significantly lower concentration of L-carnosine compared with that required for the drug alone. Interestingly, we did not observe a significant increase of efficacy when Carnosine6K and oncolytic adenovirus were added separately as a mix, perhaps because L-carnosine uses virus as a carrier to maximize cell entry, as shown in *in vitro* cell viability assay. It has been shown that poly-lysine linked to several different peptides and MHC-I restricted did not affect the cell killing activity of the virus highlighting the specific role of L-carnosine in our system.

In addition, molecular analysis performed on HCT-116 and A549 cell lines demonstrate that the oncolytic virus coated with modified L-carnosine improves the antitumor effect respect to virus or L-carnosine alone.

This synergy was mainly based on enhanced autophagy and on expression of Hsp27. Indeed, it has been reported that Ad5 induces cell lysis through autophagy and that viruses may also use autophagy-related vacuoles as a means for new progeny to exit the infected cell (27). In addition, the combination of an adenovirus with an autophagy inducer correlates positively with virus replication, improving the efficacy of Ad-mediated oncolysis. Therefore, we explored whether the oncolytic adenovirus loaded to L-carnosine, a mimic of rapamycin, improves antitumor effects in cancer cells (16). LC3II conversion performed in HCT-116 and in A549 cells demonstrates that the complex increases autophagy promoting virus replication only in colon cancer cells (HCT-116). In agreement with these results, we have found only in HCT-116 cell line, a significant increase of viral particles, by qPCR, after

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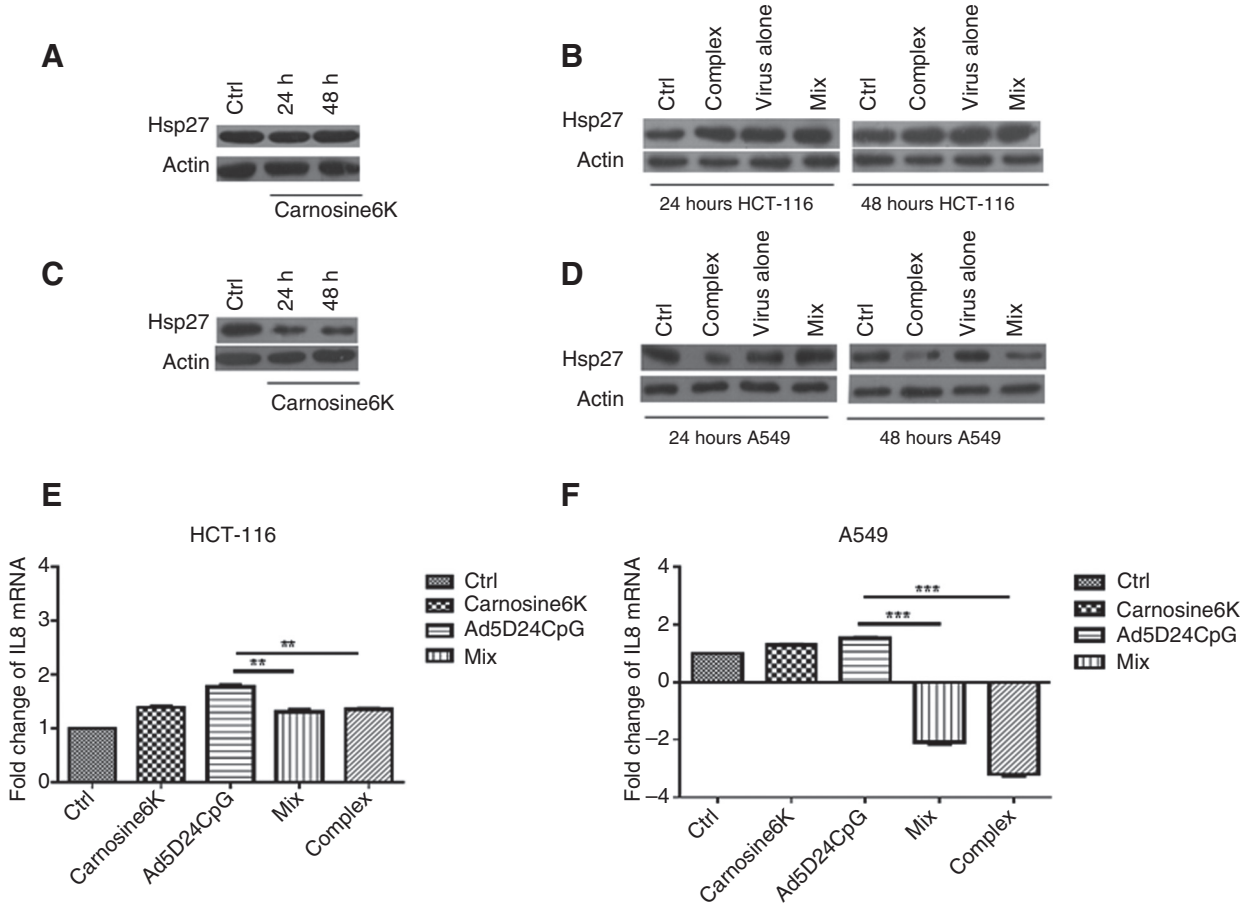

Figure 6.
Ad5D24CpG–Carnosine6K affects Hsp27 expression, modulating IL8 mRNA levels in A549 cells. A–D, Western blot analysis of HCT-116 and A549 cells treated with Carnosine6K, Ad5D24CpG, complex, or mix. Cells were harvested at 24 and 48 hours posttreatment or infection and analyzed for expression of Hsp27. Ctrl is the control sample untreated. β-Actin was used as a loading control. E and F, IL8 mRNA levels measured by real-time PCR in total RNA preparation from HCT-116 and A549 cells, after Carnosine6K treatment or postinfection with Ad5D24CpG, complex, or mix respect to untreated control sample. Primer sequences are described in Materials and Methods. Reference mRNA was that of actin. Data shown are representative results from a total of three separate experiments. Data are presented as mean ± SD for each group. **, $P < 0.01$; ***, $P < 0.001$.

complex infection. Despite the autophagy is quite evident in A549, we did not observe any differences in complex infection respect to virus alone or mix. In this cell line, we suppose that the autophagy is associated to adenovirus infection probably improved by its CpG islands. Indeed, some authors previously reported that CpG motifs might induce autophagy in human tumor cell lines by TLR9-dependent mechanism (28). The HSP expression seems to have a crucial role not only in tumor cell survival and proliferation but also in viral replication and in mediating the viral infection signaling. Actually, several studies supported the idea that cells with higher HSPs expression might have more favorable environment for virus replication. Indeed, HSP overexpression may change the level of some genes, which are responsible for adenovirus life cycle in cells such as adenovirus receptor (CAR), which affects the infection and replication of adenovirus. It has been reported that HCT-116 cells have high expression of Hsp27 (29) and we found that Hsp27 was more expressed in HCT-116 compared with A549 cell line (unpublished observations; Western blot analysis conducted in our laboratory). The basal expression of Hsp27 might favor the environment for virus replication and also exert antiapoptotic function, helping the virus to form new virus particles in an early phase of complex activity. These results are in agreement with the hypothesis that HSP expression enhances oncolytic effect of replicative adenovirus (30). In A549 cells, instead, we found a reduction of Hsp27 protein level after complex infection. In this cell line, we did not observe any difference in viral DNA replication. In addition, according to the assay for infectious titer, in HCT-116 cells, we found a higher number of viral particles after the Ad5D24CpG infection with respect to A549. On the basis of these results, we supposed that the adenovirus enters into the HCT-116 cell line with higher efficiency or that this cell line has more favorable environment for virus replication.

It is noted that in lung tumor cells, Hsp27 expression is correlated to resistance to apoptotic cell death (21). Consequently, we hypothesize that the antitumor effect of complex in A549 can be attributed, partially, to Hsp27 reduction. In addition, it has been evaluated also in IL8 mRNA levels, because a recent study strongly suggested that Hsp27, p38, and NF-kB-p65 form a signalosome in virus-infected cells influencing cytokines expression. It has been demonstrated that a siRNA of Hsp27 is able to disrupt this association reducing the IL8 expression (20).

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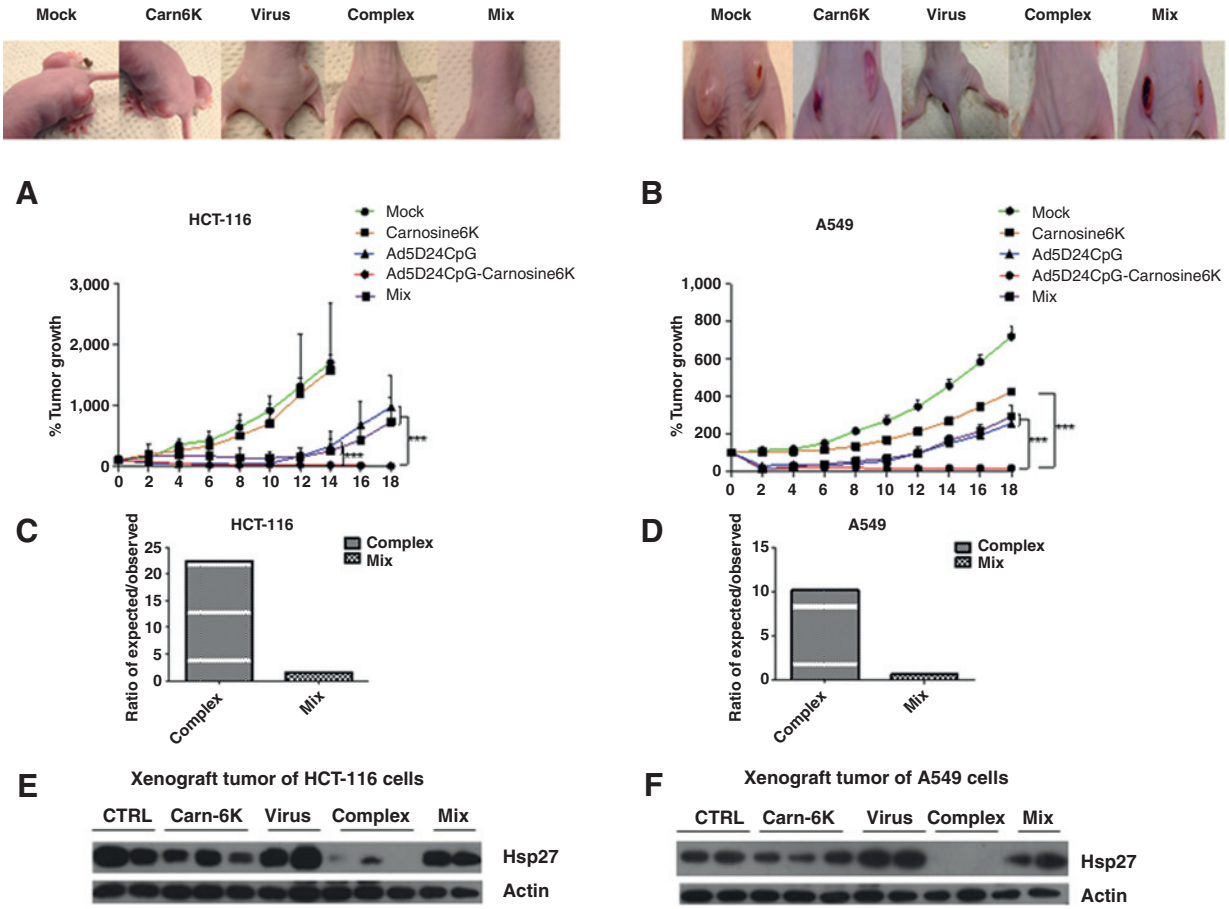

Figure 7.

In vivo efficacy of Carnosine6K (Carn6K)-coated virus. A and B, nude BALB/c bearing A549 and HCT-116 tumors were treated intratumorally with PBS or with 1×10^8 VP/tumor of Ad5D24CpG or AdD24CpG-Carnosine6K virus on days 0, 2, and 5. Tumor growth was measured over time. C and D, the assessment of therapeutic synergy was calculated with FTV method. Observed FTV (mean tumor volume experimental)/(mean tumor volume control). Expected FTV (mean FTV of Carnosine6K) × (mean FTV of virus). A ratio >1 indicates a synergistic effect, and a ratio <1 indicates a less than additive effect. E and F, expression of Hsp27 in protein extracts from xenograft tumors originated by HCT-116 and A549 cells was analyzed by Western blot analysis. β-Actin was used as a loading control. Data are representative results from a total of three separate experiments. Data are presented as mean ± SD for each group.

Endogenous expression of IL8 has been found in various human cancers, including AML, B-cell CLL, breast cancer, colon cancer, cervical cancer, gastric cancer, Hodgkin lymphoma, and ovarian, prostate, and lung cancers. Evidence shows that IL8 biologic activity in tumors and the microenvironment may contribute to cancer progression, and in other circumstances to induce antitumor response. This biologic response may be different in different types of human cancers (31). We found a significant decrease of IL8 mRNA only in A549 cells, a cell line which overexpresses EGFR. IL8 is involved in tumor cell proliferation via EGFR, and the IL8 decrease might explain the better antitumor effect of complex (32, 33). In the end, we investigated the *in vivo* efficacy of the complex in human colon cancer and lung cancer xenograft model. It has been demonstrated that the intraduodenal administration of L-carnosine decreases splenic sympathetic nerve activity (splenic-SNA) and inhibits the proliferation of HCT-116 cell line implanted into athymic BALB/c nude mice. *In vivo* data revealed that 1 mg/mL of L-carnosine solution given in the drinking water from 6 to 22 days inhibited tumor proliferation (34). Starting from these results, we evaluated that the intratumoral administration of the complex resulted in a clear tumor reduction and most of the tumors were fully eradicated within 18 days after the virus injection. In tumor growth follow-up studies, we observed that tumor growth of mice treated with complex was significantly suppressed compared with the mice treated with virus alone. Surprisingly, we also found that the expression of Hsp27 was dramatically reduced in both xenograft tumors after intratumoral administration of the complex. This result represents a promising indication, because recent studies suggest the possibility of Hsp27 inhibition for molecular target in cancer therapy. Molecular analysis is preliminary; however, it adds something new in the understanding of the molecular mechanism underlying the development of an innovative methodology. In conclusion, the results described in this study encourage the use of Ad5D24CpG–Carnosine6K for new therapeutic protocols for the treatment of cancer and in particular human colon cancer and lung cancer. Moreover, the use of oncolytic adenoviruses loaded with active drugs could be used as a novel drug delivery system for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Garofalo, B. Iovine, L. Kuryk, C. Capasso, M.A. Bevilacqua
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Other (conceived the idea and supervised Dr. Garofalo for this work): V. Cerullo

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