Leukocytes and drug-resistant cancer cells are targets for intracellular delivery by adenoviral dodecahedron

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Abbreviations: Ad Dd, adenoviral dodecahedron; Ad3, adenovirus type 3; BBB, blood-brain barrier; DOX, doxorubicin; FBS, fetal bovine serum; FSC, forward scatter; HCC, hepatocellular carcinoma; HSPGs, heparan sulphate proteoglycans; IC_{50}, half maximal inhibitory concentration; i.t., intratumoral; i.v., intravenous; MDR, multidrug resistance; MFI, mean fluorescent intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CNS, central nervous system; Pb, penton base; P-gp 1, P-glycoprotein 1; p.i., post injection; p/sec/cm^2, number of photons per second, per square centimeter; RBC, red blood cells; SSC, side scatter; WBC, white blood cells; VLPs, virus-like particles

ABSTRACT WORD COUNT: 149
TEXT WORD COUNT: 4972
NUMBER OF REFERENCES: 42
NUMBER OF FIGURES: 8
NUMBER OF TABLES: 1
NUMBER OF SUPPLEMENTARY FILES: 1

Funding: This work was supported by the National Science Centre (Poland) decision DEC-2013/09/B/NZ3/02327 and in part by Association Espoir Isere Contre Le Cancer and by association Gefluc Grenoble Dauphiné-Savoie.

The authors declare no competing financial interests

Abstract

One of the major factors limiting the effectiveness of cancer chemotherapy is inefficient drug delivery. Systems enabling efficient delivery and enhanced intracellular uptake, appear particularly promising in this respect. Virus-like particle, adenoviral dodecahedron (Dd), employs receptor-mediated endocytosis for cell penetration and is able to deliver intracellularly dozens of cargo molecules attached to one particle. We focused on studying Dd properties in the context of cancer treatment, showing that intratumoral injection of Dd, assessed in mouse xenograft model, results in vector accumulation in tumor without spreading in off-target organs. Furthermore, we demonstrated that Dd is a promising vector targeting leukocytes and drug-resistant cancer cells. Dd uptake by human blood cells analyzed *in vitro* indicated the preference for leukocytes in comparison to red blood cells and platelets. Furthermore, internalization of Dd-doxorubicin conjugate by drug-resistant cells leads to increased nuclear accumulation of doxorubicin and significant enhancement of cytotoxicity against target cancer cells.

Keywords: Adenoviral dodecahedron; virus-like particle; drug delivery, drug resistance, *in vivo* imaging

Background

The main limitation of cancer chemotheraphy is that anticancer agents are nonselective and often toxic for healthy cells. Moreover cancer cells develop resistance to chemotherapy that could not be overcome by enhancement of drug dose, because the amount of chemotherapeutic that can be administered is restricted by its side effects such as cardiotoxicity in case of anthracyclines. The improvement of cancer treatment efficacy and reduction of detrimental side effects to normal tissues may be achieved through the application of drug loaded nano-delivery systems. Currently, there are a number of nanoparticle therapeutics, that have been approved for clinical use, either by the Food and Drug Administration (FDA) in the United States, or the European Medicines Agency (EMA) in the European Union. Majority of them are liposomal systems encapsulating an anticancer drug, such as polyethylene glycol (PEG) functionalized liposomal doxorubicin, that was the first cancer nanomedicine approved by FDA in 1995, or liposomal daunorubicin approved by FDA in 1996. Also nonliposomal preparations such as albumin-bound paclitaxel and polymeric particles, such as methoxy-PEG-poly[DL-lactide]taxol, were launched. All approved delivery systems target tumors passively via the enhanced permeability and retention effect (EPR).[1] These formulations are less toxic than conventional therapies but are still associated with adverse effects such as stomatitis and palmar-plantar erythrodysesthesia for PEGyleted liposomal DOX[2] and sensory neuropathy and nausea for albumin bound paclitaxel.[3] Thus, there is a need for development of new safe drug nano-delivery systems with improved biodistribution, pharmacokinetic properties and active targeting.

Virus-like particles (VLPs) are an attractive alternative to chemically synthesized nanoplatforms in development of novel carriers for targeted drug delivery, as they do not demonstrate limitations such as toxicity of metal particles, low stability of liposomes nor structural heterogeneity of polymer-based nanoparticles.[4] Adenoviral dodecahedron (Ad Dd),

the symmetrical, small (28 nm) and non-infectious VLP, endowed with extraordinary ability for intracellular penetration, is promising vector-candidate for therapeutic development. The particle is composed exclusively of 12 copies of the pentameric viral protein, adenovirus type 3 (Ad3) penton base (Pb), one of two capsid proteins responsible for virus cell entry. Dd retains usage of receptors, αvβ3 and αvβ5 integrins, recognized by Pb within Ad particle.[5] In addition, Dd recognizes heparan sulphate proteoglycans (HSPGs) that do not serve as receptors for Ad3.[6,7] Importantly, integrins αvβ3 are abundantly expressed on angiogenic endothelial cells and some tumor cells but not on resting endothelial cells of most normal organs.[8,9] The presence of 60 RGD motifs responsible for the interaction with αv integrins, suggests Dd affinity to cancer cells. Therefore, our current work is focused on investigation of Dd properties in the context of application in cancer treatment. Dd employs the mechanism of receptor-mediated endocytosis for cell entry and has the ability to escape endosomes before reaching lysosomes, which results in cytoplasmic delivery of cargo molecules in a functional form.[10-12] The chemical and genetic modifications allow for covalent attachment of cargo molecules or their insertion into particle structure without disrupting VLP integrity or penetration properties.[13-15] The usefulness of Dd as a carrier for conjugated small molecules was already proven by targeted delivery of anticancer agents to hepatocellular carcinoma (HCC) tumor in animal model, leading to inhibition of tumor growth.[14] The main obstacle associated with the use of VLPs is potential immunogenicity and toxicity, however, our previously published data indicated slow build-up of the humoral response against Dd as well as the lack of acute toxicity of the vector upon repetitive administration of Dd conjugates.[14]

In the present work we analyzed delivery of doxorubicin (DOX) conjugated to Dd (Dd-DOX) in comparison to free DOX. It was assessed in multidrug-resistant (MDR) human uterine sarcoma MES-SA Dx5 cells, for which we confirmed the expression of αvβ3 and αvβ5 integrins involved in Dd internalization. The results of cytotoxicity tests attested the ability of

Dd for circumvention of the P-glycoprotein 1 (P-gp 1) -mediated multidrug resistance mechanism. We demonstrated that efficient uptake of Dd-DOX conjugate in MES-SA Dx5 cells leads to accumulation of DOX in cell nucleus and significantly enhances drug cytotoxicity in target cells. Since the knowledge of carrier tropism is essential for precise therapeutic application, we investigated two routes of Dd administration, intravenous and intratumoral. This study was performed in mouse xenograft model using *in vivo* fluorescence imaging. Based on the results, concerning vector accumulation in tumor and tumor-draining lymph nodes without spreading in off-target organs after intratumoral injection, we consider local application as the optimal route of administration. Furthermore, we demonstrated the accumulation of Dd in animal skin after intravenous administration which is consistent with results showing efficient penetration of primary dermal fibroblasts *in vitro*. The analysis of *ex vivo* interactions of Dd with human blood cells, provided information on preferential leukocytes targeting. Additionally, this paper contains a description of lyophilization methodology for long-term storage of the VLP.

Methods

Section containing methods concerning:
Materials
Cell lines and culture media
Vector preparation
Dd labeling
Dd imaging in mice xenograft model
Dd uptake by human dermal fibroblasts
Dd uptake by human blood cells
Detection of αvβ3 and αvβ5 integrins in human sarcoma cells
Cellular uptake of Dd in drug resistant cells
Corrected total cell fluorescence measurement
Conjugation of doxorubicin to dodecahedron particles

Cellular uptake of Dd-DOX conjugate
MTT cell viability assay
Dd lyophilization
Electron microscopy analysis
is placed in Supplemental materials.

Results

In vivo Dd-AF680 distribution

We applied non-invasive fluorescence tomography in xenograft model of mice bearing subcutaneous melanoma tumor for identification of the best route of Dd administration for optimal tumor accumulation and limited non-specific toxicity of anticancer agents delivered by VLP. We monitored tumor uptake of Dd labeled with Alexa Fluor 680 (Dd-AF680), either after intravenous (i.v.) application, which is the most common method of chemotherapy administration or after intratumoral (i.t.) injection. *In vivo* imaging after i.v. administration revealed the presence of uniform fluorescence signal across mice body visible 5 h p.i, which disappeared almost completely after 24 h p.i. (Figure 1, *A*). The uniform signal masked the fluorescence from tumor (5 h p.i.), which was revealed by *ex vivo* fluorescence measurements (Figure 1, *B* and *C*). Intravenous injection resulted in a spread distribution in off-target organs. *Ex vivo* imaging at 5 h p.i. demonstrated the strongest signal in skin and liver, and a weak signal in tumor and organs such as bladder, spleen, kidneys, adrenal, fat, lungs, uterus and guts (and a nonspecific auto-fluorescence in the stomach) (Figure 1, *B* and *C*). Dd-AF680 was also detected in isolated sciatica-, inguinal- and axillary lymph nodes (Figure S1, *A*), which indicates that Dd entered into lymphatics after extravasation from the vascular system to interstitial areas of several organs. Within 24 h the particle has been eliminated from all organs except liver, displaying weak fluorescent signal. Dd accumulation in liver upon systemic application was expected, since it is the usual clearance pathway for nanoparticles, in which the important role plays the uptake by the endothelial cells of liver sinusoids.[16]

However, the uptake by the liver did not compromise Dd distribution throughout the mice body, which resulted in the preferential accumulation of VLP in animal skin. We demonstrated only limited Dd localization and retention in mice melanoma tumors upon intravenous injection (Figure 1), therefore we further analyzed the effect of direct intratumoral application on the distribution of VLP. Tracking of Dd upon i.t. injection demonstrated accumulation of Dd-AF680 in tumor at 5 and 24 h p.i. (Figure 2, *A*). *Ex vivo* imaging and analysis of isolated organs confirmed persistence of Dd-AF680 in tumors at 5 and 24 h p.i., without localization in off-target organs, except residual signal in liver and skin (Figure 2, *B* and *C*). Importantly, the particle was present for 24 h p.i. in tumor-draining lymph nodes, being the first site of metastasis in most types of cancer (Figure S1, *B*). Of note, it appears that Dd is unable to pass the blood-brain barrier (BBB), as no signal was observed upon *ex vivo* imaging of isolated brains (Figure 1, *B* and 2, *B*). It is consistent with our results of *in vitro* studies, in which either co-culture endothelial BBB model or MDCKII-MDR1 BBB surrogate model were used (results not shown).

Dd uptake by human dermal fibroblasts

The results of *in vivo* imaging after intravenous injection of Dd showed particle accumulation in animal skin (Figure 1, *B* and *C*). Therefore, we investigated the ability of Dd to penetrate dermal fibroblasts, the main cells of the skin dermis. Our results confirmed that Dd applied to human primary dermal fibroblasts, easily penetrates them. Internalization of Dd-AF647 increases with the rising Dd concentrations reaching over 90% and almost 100% of transduced cells for 10 and 30 μg/ml Dd, respectively (Figure 3).

Dd uptake by human blood cells

Targeting ability of Dd toward human blood cells is important factor for its application as drug carrier. Therefore fresh total blood was evaluated for transduction by Dd labeled with Alexa Fluor 647 fluorescent dye (Dd-AF647), to analyze target cell types in a single tube by

flow cytometry. When blood samples were treated for 1 h with Dd-AF647 (range 0.7 - 12.2 pmoles) affinity to red blood cells and platelets was quite low in comparison to leukocytes (Figure 4, *A* and *C*). Indeed, percent of transduced cells were similar at 0.7 and 1.4 pmoles of Dd but the mean fluorescent intensity (MFI) was high in leukocytes when compared to red blood cells and platelets. When exposed to higher concentrations of Dd, the percent of transduced cells increased in all cells, but with a superior efficiency on leukocytes, close to 50% platelets and RBC were transduced at the maximum, whereas it reached 85% on leukocytes. More notably, at 12.2 pmoles Dd treatment of blood, leukocyte MFI reached~45 times the values observed for RBC and platelets (Figure 4, *A* and *C*).Since the MFI is proportional to the number of fluorescent molecules, we can conclude that Dd preferentially penetrates leukocytes. We also analysed how the Dd transduction depends on leukocytes subtypes (Figure 4, *B*). We focused on T-cells since they are lymphoid cells and are easily identified by the specific expression of CD3 from their receptor. We also explored Dd transduction on cells specialized into foreign molecules capture. We therefore analyzed $CD33^{+}$ cells since this protein is expressed on membrane of myeloid lineage cells (including polynuclear cells, phagocytes and subtypes of dendritic cells) and CD14 that is expressed, in blood, mainly but not exclusively, by monocytes. All leukocytes were efficiently transduced by Dd and no distinctive amount of fluorescent dye was detected on different leukocytes subtypes (Figure 4, *B*). Despite leukocytes representing a minor fraction of blood cells (about thousand times less cells compared to RBC and about fifty times less than platelets), we observed that the affinity of Dd in blood is highly in favor of leukocytes, independently of their subtypes.

Dd uptake by drug-resistant cancer cells

Dd, similarly to adenoviruses, attaches to cell membrane and penetrates cells by receptor-mediated endocytosis, therefore we probed if it would be internalized by drug-resistant cells,

overexpressing P-glycoproteins, drug efflux pumps for xenobiotic compounds with broad substrate specificity. For this purpose we had chosen human uterus sarcoma cell line MES-SA and its multidrug-resistant counterpart, MES-SA Dx5. Since, to the best of our knowledge, the expression of Dd receptors on human uterus sarcoma cells was not reported hitherto, we started from the verification of their presence. The fluorescence microscopy imaging with the use of specific antibodies confirmed the presence of αvβ3 and αvβ5 integrins, which are involved in Dd internalization, on the surface of MES-SA and MES-SA Dx5 cells (Figure 5, *A*). The confocal microscopy analysis, performed after 2 hour incubation of MES-SA and MES-SA Dx5 cells with 10 μg of Dd, demonstrated lack of differences in the amount and intracellular localization of the vector (Figure 5, *B* and *C*). While analyzing Dd penetration, we observed that particle endocytosis increased up to 2 h (Figure 5, *B* and *C*), while the amount of intracellular Dd diminished at later time points (data not shown).

Doxorubicin conjugation to Dd particle

We wanted to verify if conjugation of doxorubicin to Dd (Dd-DOX) improves antibiotic delivery to drug-resistant cancer cells. In the preparation of Dd-DOX conjugates we used the NHS-ester-activated adipic acid as a crosslinker for coupling lysine residues of Dd with the primary amine groups of doxorubicin. Doxorubicin concentration in Dd-DOX conjugates, determined by fluorescence intensity measurement with the standard curve for DOX and UV-vis spectra of DOX and Dd-DOX, was 3 μM. The average Dd loading with drug was estimated to about 90 molecules of DOX per one molecule of vector.

Intracellular distribution of Dd-DOX and free DOX

Cell entry of Dd-DOX conjugate (and free DOX) was monitored by confocal microscopy. Figure 6 shows subcellular localization of free DOX and Dd-DOX in MES-SA and MES-SA Dx5 cell lines four hours after application. The treatment with free DOX resulted in the classical image of nuclear drug localization in MES-SA cells, whereas only a negligible

fluorescence of DOX (green) can be observed inside MES-SA Dx5 cells, which is most likely related to the expression of P-gp protein in these cells.[17] All MES-SA and MES-SA DX5 cells were found to be transduced with Dd-DOX, as indicated by the presence of Dd (red stain) in cytoplasm of each cell. In both cell lines similar intracellular localization was observed for DOX, delivered in the form of conjugate, with DOX fluorescence signal present in the nuclei as well as in the cytoplasm of transduced cells. The presence of DOX molecules distributed in cytoplasm suggests that they were still attached to the carrier, while the majority of DOX was already released from conjugate and delivered into the nucleus.

Cytotoxicity of Dd-DOX conjugate

According to our results of MTT assay, the application of the vector alone did not affect the viability of either MES-SA or MES-SA Dx5 cells (Figure 7, *A*), however, delivery of Dd-DOX conjugate resulted in diminished, concentration-dependent viability of both, drug-resistant and drug-sensitive cells after incubation for 48 hours. After treatment with equal concentrations of doxorubicin in a form of conjugate in comparison with a free form of drug, the IC_{50} (half maximal inhibitory concentration) value, according to MTT assays, was on average fourfold lower for Dd-DOX conjugate than that for free DOX for MES-SA, and approximately five fold lower for MES-SA Dx5 cells (Figure 7, *B*, Table 1).

Dd storage conditions

For the further therapeutic uses of Dd the elaboration of method for convenient particle storage and transportation was needed. We proposed simple protocol for Dd lyophilization for prolonged storage. Electron microscopy and In-cell ELISA analyses of water-reconstructed samples indicated that Dd purified in two-step protocol based on size exclusion and anion exchange chromatography[18] can be frozen and lyophilized in the presence of sodium chloride and cryoprotectants (sucrose and mannitol), without inducing aggregation, losing structural integrity or penetration capabilities (Figure 8). Of note, even preparations lyophilized in the

absence of cryoprotectants after reconstitution in water still contained a large proportion of well-formed dodecahedra, although a significant amount of free pentameric bases was observed (data not shown). Lyophilized Dd can be stored at temperatures up to 37 °C, which means that protein could be transported and used without refrigeration (Figure 8, *A*). Moreover, we observed no substantial difference in cell penetration capabilities before and after Dd lyophilization and storage at indicated temperatures (Figure 8, *B*). Clearly, the presence of cryoprotectants did not affect cell internalization.

Discussion

Our aim was the evaluation of adenoviral dodecahedron tropism and identification of VLP properties important for its development as drug delivery platform. Selection of suitable route of administration is essential for accurate planning of dodecahedron application in cancer treatment. The low tumor uptake, observed upon intravenous injection, suggests limited usefulness of this route of administration for Dd-mediated delivery to melanoma tumor. Our previous studies showed that when applied intraperitoneally, Dd conjugates carrying anticancer agents efficiently targeted tumors in orthotopic hepatocellular carcinoma rat model.[14] After intraperitoneal injection Dd conjugates entered into the mesenteric blood circulation and then were carried via the portal vein directly to liver finally accumulating in HCC tumor but not in healthy tissues.[14] This discrepancy may be explained by differences in the angiogenesis and permeability of blood vessels between xenograft model used for intravenous administration and allograft model used for intraperitoneal injection.[14] As opposed to intravenous administration, the intratumoral injection resulted in long-lasting persistence of the vector in melanoma tumor without spreading in off-target organs. Our results suggest that intratumoral application may decrease eventual drug cytotoxicity in healthy tissues, enabling usage of higher concentrations of Dd-drug conjugates. To conclude, our previous and recent results, we consider Dd suitability for local application.

Surface functionalization of nanoparticles with specific ligands enables not only specific cellular targeting but also promotes crossing biological barriers such as a BBB.[19] It is a common approach, for example in the case of Cowpea mosaic virus (CPMV) VLPs conjugation with antibodies against Human Epithelial Receptor-2[20] or with transferrin,[21] which was also used for functionalization of zolendronic acid (ZOL)NPs.[22] The improved Dd targeting of tumor upon intravenous application may be achieved by particle modification, for example with short homing peptides, which specifically recognize and bind to surface molecules on tumor cells or tumor vasculature.[23] It is worth mentioning, that Dd structural modification has already been validated for insertion of antigen peptides into external particle positions without disrupting its integrity, nor penetration properties.[15] Observed Dd lack of ability to pass through the BBB excludes the possibility of using non-targeted dodecahedron as a vector for factors acting on the central nervous system (CNS), thereby enabling the safe therapeutic applications of the particle, without possible adverse side effects on CNS.

Dd distribution through the mice body resulted in the preferential accumulation of VLP in animal skin. The interaction with HSPGs present at the surface of cells and in the extracellular matrix of skin may be involved in Dd affinity to this organ, while similar tropism was not observed for the Ad3;[24] it is worth to emphasize that Ad3 does not use HSPGs as receptors for internalization. Together, Dd tropism to animal skin observed upon i.v. application and ability for efficient penetration of dermal fibroblasts observed *in vitro* suggests the utility of VLP as a carrier for delivery to skin dermis. Importantly, the ability to penetrate primary cells, first shown for the primary human astrocytes[11], is an important attribute of the vector for its potential application, since primary cells are difficult to transfect using electroporation or chemical transfection reagents.[25] Thus, the use of Dd offers a new solution to this challenge.

Previously reported results, on Dd stability in human serum,[13] prompted us to investigate carrier affinity to blood cells. Interactions with blood cells may have important consequences for undesirable vector elimination from the blood stream and its degradation, as it was demonstrated for recombinant adenovirus type 5 (Ad5) gene therapy vectors.[26 27] It was also shown for Ad3 that surface interaction with platelets and granulocytes is mediated by binding to desmoglein 2 (DSG-2, the receptor of adenovirus fiber),[28] which, however, cannot function as the attachment receptor for fiber-less dodecahedra used in our studies. Importantly, known receptors essential for Dd internalization, are present on the surface of freshly isolated circulating leukocytes in low amounts.[29] It seems that cell surface molecules, other than already identified receptors, may be involved in WBC recognition since the low level of αvβ3 integrins and HSPGs expressed by small fraction of cells might be not sufficient to support efficient penetration of about 85% leukocytes demonstrated in current study. It is worthwhile to mention that Ad2 attachment to and infection of monocytic cells is facilitated by the described interaction of penton base protein with $\alpha_M\beta2$ integrin.[30] Within $\alpha_M\beta2$ integrin, the αMI-domain is responsible for the ability of multiligand binding. Based on the algorithm that predicts the αMI-domain binding sites in the $\alpha_M\beta2$ ligands[31] and analysis of Ad3 Pb sequence and structure[32] we predict that the relevant recognition motif is localized in the region of externally exposed variable loop of Ad3 Pb. Mechanistic studies on the uptake of Dd in human leukocytes could deepen the understanding of this ability. However, the most important point is the distinct Dd transduction efficiency for white blood cells, since it could lead to the use of this vector for the transport of active molecules targeting leukocytes, for example as a putative drug carrier targeting leukaemia cells, in particular for *in vitro* testing of potential anticancer agents intended to treat leukocyte disorders. The suitability of Dd for such application is supported by the lack of cytotoxicity of this VLP in human peripheral blood mononuclear cells.[15]

A major problem facing current cancer treatment is tumor resistance to chemotherapy. The most common mechanism of resistance is mediated through the drug efflux pump, P-gp 1 protein, that expels membrane-embedded exogenous molecules.[33] Various delivery systems based on mesoporous silica NPs,[34] liposomes[35,36]and micelles[37-38] have been developed to circumvent drug resistance of cancer cells. Our approach is based on the remarkable cell penetration capability of Dd nanoparticles. While free doxorubicin enters cell by a passive diffusion across the plasma membrane[39] and it may be expelled by Pgp-1 from drug-resistant cells,[40] Dd-DOX conjugate penetrates cells via receptor-mediated endocytosis, which conceivably does not allow doxorubicin recognition by the efflux pumps that are localized in the plasma membrane.[41] Confocal microscopy images of uterine sarcoma MES-SA cells treated with free DOX are consistent with literature data on rapid intercalation of DOX within nuclear DNA[42] in sensitive cells and significantly reduced nuclear uptake in case of cells expressing P-gp efflux pumps. Treatment of MES-SA and MES-SA Dx5 drug-resistant cells with Dd-DOX resulted in the high nuclear and, rather low, cytoplasmic localization of DOX carried by Dd (Figure 6). DOX fluorescence in cytoplasm is associated with drug conjugation to Dd whereas DOX accumulation in the nucleus must be due to its release from conjugate, because Dd does not enter nucleus[10]. The nuclear accumulation of DOX 4 hours post application of conjugate suggests rapid proteolysis of the vector, which might result in liberating of peptides containing DOX, probably able to easily translocate to the nucleus. Indeed, the dodecahedric vector was observed to undergo rapid proteolysis inside transduced cells.[13] Consistently with the enhanced drug delivery to MES-SA and MES-SA Dx5 cells, Dd-DOX represents higher cytotoxicity than free DOX in both cell lines, with more pronounced effect exerted against drug resistant cells (Figure 6, Table 1). The current results are in agreement with our previous *in vitro* studies, when we observed efficient delivery of Dd-DOX conjugate to human HeLa and rat Morris hepatoma MH-3924A cells, with the

similar pattern of DOX intracellular localization.[14] Reported previously cell growth inhibition upon Dd-DOX treatment of drug sensitive HeLa cells and two liver cancer cell lines: rat Morris hepatoma MH-3924A and human hepatocellular carcinoma PLC/PRF/5, was achieved with 5 fold lower amount of doxorubicin in conjugate, when compared to application of free drug. These data are consistent with presented here results of cytotoxicity tests performed on uterine sarcoma cell lines. On the grounds of our results we consider Ad3 Dd to be a promising vector for intracellular delivery that enables to overcome drug-resistance in cancer cells.

Additionally this paper contains a description of simple and useful procedure for the vector long-term storage at broad spectrum of temperatures, without disrupting vector structure and properties. Thus we believe that Dd meets the important requirement for drug carriers, which is convenient storage and transportation.

Figure captions

Figure 1. Dd-AF680 localization after systemic application. Nude mice were injected intravenously with 33 µM (200 µl) Dd-AF680. A) *In vivo* fluorescence imaging of Dd-AF680 at T0 (before injection), at 5 h and 24 h post injection (p.i.). T - tumor, LN - lymph nodes, p/sec/cm^2 - number of photons per second, per square centimeter. B) *Ex vivo* fluorescence imaging of isolated organs and tumors 5 and 24 h p.i. C) *Ex vivo* fluorescence analysis of isolated organs and tumors for Dd-AF680 injected and control (Ctl, non-injected) mice, at indicated times p.i.

Figure 2. Dd-AF680 localization after intratumoral injection. Nude mice were injected with 8.25 µM (50 µl) Dd-AF680. A) *In vivo* fluorescence imaging of Dd-AF680 at T0 (before injection), 5 h and 24 h post injection (p.i.). T - tumor, p/sec/cm^2 - number of photons per second, per square centimeter. B) *Ex vivo* fluorescence imaging of isolated organs and tumors 5 and 24 hp.i. C) *Ex vivo* fluorescence analysis of isolated organs and tumors for Dd-AF680 injected and control (Ctl, non-injected) mice at indicated times p.i.

Figure 3. Flow cytometry analysis of Dd uptake in primary dermal fibroblasts. Fibroblasts were incubated with 10 or 30 µg/ml of Dd-AF647 respectively for 1 h at 37 °C. A) A bar graph representing mean values and standard deviations of summarized data from three separate set of experiments and triplicate measurements per each experiment. B) A representative analysis for one experiment. On histograms, the percentage of cells transduced by Dd-AF647 is reported for each population as well as mean fluorescence intensity (MFI) at increased concentration.

Figure 4. Blood cells penetration. Fresh blood cells were exposed to Dd-AF647 for 1 h at 37 °C, 0.7 up 12.2 pmoles, and analyzed by flow cytometry. Platelets, red blood cells (RBC) and leukocytes were gated by their respective specific expression of CD41a, CD231a and CD45. The threshold percent gate was set-up by cell type for background on samples in the absence of Dd treatment. A) A representative analysis on a blood sample from one donor. On histograms, the percentage of cells transduced by Dd-AF647 is reported for each population as well as mean fluorescence intensity (MFI) at increased concentration. B) The analysis of detailed subpopulations of leukocytes is shown: T-cells are gated on CD3 expression, myeloid are CD33$^+$ cells and CD14$^+$cells are mainly monocytes. C) Data from four distinct donors are shown and summarized by a histogram of MFI measured on blood cells. Cell counts are

indicated in the tables (times 10^9 cells/l). On histograms, the percentage of cells transduced by Dd-AF647 is reported for each population as well as MFI at increased concentration. Data represent mean values with standard deviations for four experiments and triplicate measurements for each experiment. Data were compared by applying Student's *t*-test, which indicated statistically significant differences between leukocytes compared to RBC and platelets for all amounts of Dd from 1.4 up to 12.2 pmoles (** $P < 0.01$). For 0.7 pmoles of Dd the P value was lower than 0.05.

Figure 5. Dd penetration of drug-resistant cells. A) Fluorescence imaging of integrins αvβ3 and αvβ5 in MES-SA and MES-SA Dx5 cells. Integrins αvβ3 and αvβ5 were detected with anti-αvβ3 or anti-αvβ5, respectively and secondary Alexa Fluor 488 conjugated antibody (green), cell nuclei were stained with DAPI. Scale bar - 10 μm. B) Confocal microscope images of Dd internalization to MES-SA and MES-SA Dx5 cells. Dd entry kinetics was measured at indicated time points, between 0 - 120 min. Intracellular Dd was recognized with rabbit polyclonal anti-Dd serum and secondary anti-rabbit antibody conjugated with Alexa Fluor 488 (green), cell nuclei were stained with DAPI (blue). Scale bar - 10 μm. C) Corrected total cell fluorescence (CTCF) measurement for MES-SA and MES-SA Dx5 cells related to indicated times of incubation with Dd. Data represent mean ± s.d. for five measurements.

Figure 6. Intracellular uptake of Dd-DOX and free DOX. MES-SA and MES-SA Dx5 cells were incubated with 2 μM Dd-DOX conjugate or free DOX for 48 hours, respectively. Intracellular Dd was recognized with rabbit polyclonal anti-Dd serum and secondary anti-rabbit antibody conjugated with Alexa Fluor 647 (AF647). Images were obtained with Zeiss Axio Observer Z1 inverted microscope. The confocal microscope settings were the same between specimens. DOX and AF647 fluorescence was recorded with software-added green and red pseudo color, respectively, using Fiji software.

Figure 7. Cytotoxic effect of Dd-DOX conjugate determined by MTT assay. A) MES-SA and MES-SA Dx5 cells were treated with Dd (6 μg), Dd-DOX (2, 4, 6 μg delivering 50, 100, 150 nM DOX respectively) and free DOX (50, 100, 150 nM). Cell viabilities were compared to their respective non-treated controls. Data were compared by applying Student's *t*-test, which indicated statistically significant differences between DOX and Dd-DOX treated cells (* $P < 0.05$, ** $P < 0.01$). Data represent mean ± s.d. ($n = 3$ experiments, five wells per treatment). B) Dose–response curves for Dd-DOX conjugates and free DOX for the MES-SA (left panel) and MES-SA Dx5 (right panel) cell lines. Various concentrations of Dd-DOX (20, 50, 100,

150, 260, 390 nM) and DOX (50, 100, 150, 300, 450, 600 nM) were applied, and the viability of cells was determined by the MTT assay. Non-linear regression analysis was performed using Origin software (OriginLab Corporation, USA). The averaged percentage corresponding to viability of cells was plotted against the logarithm of the concentration of Dd-DOX conjugates and free DOX. Curves were fitted to a dose-response function according to the equation y = A1 + ((A2-A1)/(1 + 10^(logx0-x)•p)). Each value is the mean ± standard error obtained for three experiments (n = 3 experiments, five wells per treatment).

Figure 8. Dd lyophilization and storage in 20 mM HEPES buffer containing 0.1 mM EDTA, 150 mM NaCl and cryoprotectants (sucrose 0.4% v/v and mannitol 0.4% v/v). Separate 300 µg aliquots were lyophilized and stored for 10 days at indicated temperatures and reconstituted in sterile water. A) Electron microscopy analysis. Upper panel, on the left: control sample before lyophilization (Dd), on the right: sample after freezing in liquid nitrogen, stored for 10 days at -20 °C (control freezing). Lower panel – samples after lyophilization stored at indicated temperatures. Scale bar 100 nm. B) In-cell ELISA analysis of reconstituted Dd internalization to HeLa cells. Cells were incubated for 2 h at 37 °C with 4 µg of Dd. Data represent mean ± s.d. for triplicates.

Table 1

Concentration inhibiting 50% of cell growth (IC_{50}) reached after 48 h of treatment with Dd-DOX and free DOX in MES-SA and MES-SA Dx5 cells. Data represent mean values and standard deviations (SD) for three separate experiments.

Acknowledgements

We are grateful to M. Lichocka (IBB, Warsaw, Poland) for help with confocal microscopy and M. Mielecki (IBB, Warsaw, Poland) for help with statistical analysis.

Author Contributions

Designed the experiments: J.C., M.J., D.L., E.S. Performed the experiments: P.D., M.J., D.L., M.P.B., E.S., I. S. Analyzed the data: J.C., P.D., M.J., D.L., E.S. Contributed reagents/materials/analysis tools: J.C., D.L., E.S. Wrote the paper: J.C., M.J., D.L., E.S.

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