Supplemental materials

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

Marta Jedynak [1], David Laurin, PhD [2], Patryk Dolega[1], Malgorzata Podsiadla-Bialoskorska [1], Inga Szurgot, PhD [1,3], Jadwiga Chroboczek, PhD [2,1], Ewa Szolajska, PhD [1,*]

[1]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland;

[2]Université Grenoble Alpes, CNRS, Grenoble INP, TIMC-IMAG, EFS, F38000 Grenoble, France

[3]Current address: Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Nobels vag 16, SE-171 77 Stockholm, Sweden

*Corresponding author: E.S
E-Mail: ewasz@ibb.waw.pl
Phone: +48 22 5922420

Supplemental Methods

Materials

Anti-Dd polyclonal rabbit serum was prepared in laboratory. Mouse anti-integrin αvβ3 antibody, clone LM609, mouse anti-integrin αvβ5 antibody, clone P1F6 and goat anti-rabbit IgG HRP-conjugate antibody were purchased from Sigma. Alexa Fluor 488 conjugated chicken anti-rabbit IgG, Alexa Fluor 488 conjugated chicken anti-mouse IgG, Alexa Fluor 647 conjugated goat anti-rabbit IgG and Alexa Fluor protein labeling kits were purchased from Molecular Probes.

Cells and culture media

HeLa cells (ATCC® CRMCCL2™) were purchased from the American Type Culture Collection. Human uterine sarcoma cell line MES-SA (ECACC 95051030) and MES-SA Dx5 (ECACC95051031) were purchased from the European Collection of Authenticated Cell Cultures. HeLa, MES-SA and MES-SA Dx5 cells were maintained in Dulbecco's Modified Eagle's (DMEM) medium containing high glucose and L-glutamine supplemented with 10%,

15% and 15% FBS respectively (Cytogen) and streptomycin/penicillin (100 µg/ml and 100 IU/ml, respectively). Human primary dermal fibroblasts were purchased from PromoCell (Germany) and were cultivated in DMEM supplemented with GlutaMAX, streptomycin/penicillin (100 µg/ml and 100 IU/ml, respectively) and 10% FBS. Cells were used before reaching passage 12th.

Vector preparation

Dd was expressed in FlashBacGold (Oxford Expression Technologies) baculovirus expression system following the manufacturer's protocol. Virus stocks were prepared by infection of *Spodoptera frugiparda* 21 monolayer. For Dd expression, *Trichoplusia ni* (High-Five) cells (2 x 10^6 cells/ml) grown in suspension were infected with the recombinant baculovirus at multiplicity of infection 4 and cultured for 48 h. Expressing cells were lysed and Dd was purified from the supernatant by size exclusion chromatography followed by anion exchange chromatography, as described previously.[1] Dd preparations were concentrated using Amicon Ultra concentrators (Merck Millipore), protein concentration was determined by NanoDrop ND-200 UV-Vis Spectrophotometer (Thermo Scientific) and SDS-PAGE analysis in the presence of a known amount of BSA. The homogeneity of preparations were analyzed by SDS-PAGE. The assembly status of purified VLPs was analyzed by electron microscopy.

Dd labeling

Dd at a concentration of 1 mg/ml was labeled with Alexa Fluor 647 (Dd-AF647) or with Alexa Fluor 680 (Dd-AF680) using the appropriate protein labeling kits. Labeling reaction was performed according to a manufacturer protocol. The resulting conjugates were purified by the extensive dialysis against PBS. The absorbance of the conjugate solution was measured at 280 nm and 650 nm or at 280 nm and 679 nm for Alexa Fluor 647 or Alexa Fluor 680, respectively (Synergy HTi plate reader, Biotek, Winooski VT, USA). Degree of labeling (DOL) was calculated according to manufacturer's protocol.

Dd imaging in mice xenograft model

All animal experiments were conducted in agreement with the European Union guidelines and the Principles of Laboratory Animal Care (National Institutes of Health publication no. 86–23, revised 1985) and approved by the regional ethics committee (Cometh38, Grenoble). Six female NMRI nude mice (5 weeks old) weighing 24 ± 0.5 g, were purchased from Janvier (Le

Genest St. Isle, France). Mice were injected subcutaneously on the right flank with $3x10^6$ A375rvluc2 cells (human melanoma). When tumors reached 258 ± 68 mm^3, 21 days after cell injection, mice were anaesthetized (isoflurane/air 4%) and injected with Dd-AF680. Two mice were injected intravenously via the tail vein, two were injected intratumorally with 200 µl (33 µM) and 50 µl (8.25 µM) of Dd-AF680, respectively whilst two mice served as non-injected controls. The 2D fluorescence was obtained with IVIS kinetics (PerkinElmer), with excitation at 675 nm and emission at 720 nm. *In vivo* fluorescence imaging was performed before injection (at T0), at 5 h and 24h post injection (p.i.). After mice were sacrificed, the internal organs, lymph nodes, and grafted tumors were isolated at 5 and 24 h p.i. for *ex vivo* imaging. The results of fluorescence analysis are expressed as the number of photons per second, per square centimeter ($p/sec/cm^2$). In parallel, the autofluorescence of organs and tumors isolated from two control (non-injected) mice was determined.

Dd uptake by human dermal fibroblasts

Cells were seeded in 24-well plate at $3x10^4$/well in DMEM medium containing 10% FBS and streptomycin/penicillin (100 µg/ml and 100 IU/ml, respectively). Two days later medium was replaced by 200 µl PBS and Dd-AF647 (fluorophore concentration 13 µM) was added to obtain final concentrations of 0.27 to 0.81 pmoles of Dd. After 1 h at 37 °C in 5% CO_2 culture incubator, supernatant was removed, cells were washed with PBS and detached with 0.5% trypsin/EDTA for 3-4 min at 37 °C. Cells were next transferred to cytometry tubes and washed once again with PBS-10% FBS. The pellet was resuspended in 200 µl PBS for FACs analysis with FACSCanto II (BD Biosciences).

Dd uptake by human blood cells

Fresh human blood in buffered sodium citrate anticoagulant was obtained from healthy donors after informed consent. A complete blood count was performed on 200 µl by a calibrated haemocytometer. Mean cell count for 1 µl sample indicated $4.18x10^6$ of red blood cells (RBC), $4.45x10^3$ of leukocytes and $2.25x10^5$ platelets. For Dd entry assay, a portion of 1 µl of blood was diluted in 500 µl PBS, Dd-AF647 was added to obtain final concentrations of 0.7 up to 12.2 pmoles and incubated for 1 h with blood at 37 °C in the presence of 5% CO_2. Then cells were washed twice with PBS-10% FBS, pelleted for 5 min at 800 x g and resuspended in 200 µl PBS for FACs analysis with FACSCanto II (BD Biosciences). Cell types were distinguished by their size and granularity by gating on forward scatter (FSC) and side scatter (SSC) and confirmed by anti-glycophorin A (anti-CD235a, Miltenyi) for RBC,

anti-CD45, anti-CD3, anti-CD33, anti-CD14 (BD Biosciences) for leukocytes, and with anti-CD41a (BD Biosciences) for platelets.

Detection of αvβ3 and αvβ5 integrins in human sarcoma cells

MES-SA and MES-SA Dx5 cells were seeded in complete DMEM growth medium without Phenol Red on sterile poly-D-lysine-coated glass coverslips placed in the individual wells of 24-well plate at equal density ($5x10^4$ cells per well) and allowed to attach overnight. For fluorescence analysis culture medium was removed on the next day and cells were fixed with 80% cold aceton for 15 min at -20 °C, blocked with PBS 0,1% Tween 20 (PBST) containing 3% BSA, for 1 h at 37 °C and incubated overnight at 4 °C with mouse anti- αvβ3 (1:100) or anti- αvβ5 (1:1,000) antibodies respectively, rinsed thrice with PBST, incubated with the Alexa Fluor 488 conjugated goat anti-mouse IgG (1:500) for 1 h at RT, washed again thrice with PBST and stained with DAPI (5 μg/ml) for 10 min at RT. The images were captured using NikonEclipse E800 epifluorescence microscope. DAPI and Alexa Fluor 488 fluorescence was excited at 340-380 nm and 465-495 nm, and emission was measured at 435-485 and 515-555 nm, respectively. Images shown in Figure 5A were processed with Fiji (ImageJ)[2] software and were adjusted simultaneously.

Cellular uptake of Dd in drug resistant cells

MES-SA and MES-SA Dx5 cells were seeded on sterile poly-D-lysine-coated coverslips placed in the individual wells of 24-well plate at $5x10^4$/well. The next day, cells were washed 3 times with cold PBS and incubated on ice with Dd (20μg/ml) diluted in DMEM without FBS for 2 h (attachment step). Subsequently, cells were washed 3 times with cold PBS and incubated further at 37 °C in 5% CO_2 for 2 h with fresh DMEM medium supplemented with 10% FBS (synchronized entry). Cells were fixed at the indicated time points: T0 (after cell attachment), 20, 45, 90 and 120 min respectively. Cells were fixed with 100% cold methanol for 20 min at -20 °C, blocked with 5% BSA in PBS for 1 h at 37 °C and incubated for 1 h at room temperature (RT) with the anti-Dd polyclonal rabbit serum (1:1,000), rinsed with PBS, incubated with the Alexa Fluor 488 (AF488) conjugated chicken anti-rabbit IgG (1:500) for 1 h at RT, washed with PBS and finally stained for 10 min at RT with DAPI (5μg/ml). Coverslips were sealed in a closure medium (Mowiol, Fluka) and left for 24 h at 4°C. Images were collected with EZ-C1 Nikon CLSM attached to inverted microscope Eclipse TE2000 E using oil immersion objective ×60, Plan Apo 1.4NA (Nikon). DAPI and AF488 fluorescence was excited at 408 and 488 nm, and emission was measured at 430-465 and 500-530 nm,

respectively. All images were collected at 512×512 resolution and zoom 2.0. Figures were processed with EZ-C1 Viewer. Contrast of all the real-color images shown in Figure 5*B* was adjusted simultaneously.

Corrected total cell fluorescence measurement

The fluorescence intensity was determined by counting pixels using ImageJ and the corrected total cell fluorescence (CTCF) average for 5 cells for each time point was calculated using Excel and the formula: CTCF= the value of integrated density - (area of selected cell x mean fluorescence of background readings).

Conjugation of doxorubicin to dodecahedron particles

Prior to conjugation, Dd storage buffer was exchanged with 50 mM borate buffer, pH 8.5 using size exclusion column.To prepare the crosslinker the carboxyl group of adipic acid (10 mg) was activated by TSTU (O-(N-Succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (41 mg) and triethylamine (19 µl) diluted in DMSO to a final volume of 500 µl. Crosslinking reaction was performed by mixing 1 mg of doxorubicin hydrochloride (diluted in 500 µl of 500 mM borate buffer, pH 8.5) with 0.55 µl of the activated crosslinker and 1 mg of Dd (1 mg/ml) for 2 hours at room temperature with gentle shaking. Concentration of substrates was adjusted to obtain a tenfold molar excess of doxorubicin over the lysine residues in dodecahedron particle. During the reaction the pH was measured every 30 min and if needed, was adjusted to pH 8.5 with 0.1 M potassium hydroxide. The reaction was quenched by lowering the pH to 7.5 using 0.2 M acetic acid. Samples were then centrifuged for 5 min at 13 000 rpm and purified on 40K Zeba spin desalting column, according to the manufacturer's protocol (Thermo Scientific). The conjugates were stored at 4 ºC until use. The amount of doxorubicin crosslinked to Dd was evaluated spectrometrically (BioTEK Synergy HT Microplete Reader) by measuring fluorescence intensity at the excitation and emission wavelengths of 485/20 and 590/35 nm, respectively. The amount of drug in Dd-DOX preparation was estimated by reference to fluorescence obtained for a series of doxorubicin dilutions prepared in 20 mM HEPES buffer, pH 7.5, 150 mM NaCl.

Cellular uptake of Dd-DOX conjugate

MES-SA and MES-SA Dx5 cells were seeded at equal density ($4x10^4$ cells per well in 12-well chamber slide (Ibidi)) in a complete culture medium without Phenol Red and allowed to attach overnight. On the next day culture medium was replaced with 200 µl serum free

DMEM medium w/o Phenol Red containing 2 μM free DOX or Dd-DOX conjugate bearing 2 μM DOX respectively. After 3 h incubation at 37 °C the medium was removed and replaced with DMEM w/o Phenol Red containing 10% FBS and the incubation was continued for 1 hour at 37 °C. For immunofluorescence staining cells were fixed with 80% cold aceton for 15 min at -20 °C, blocked within (PBST) containing 3% BSA and for 1 h at 37 °C and incubated for 1 h at room temperature (RT) with anti-Dd polyclonal rabbit serum (1:1,000), rinsed with PBST, incubated with Alexa Fluor 647 conjugated goat anti-rabbit IgG (1:500) for 1 h at RT, washed with PBST and finally stained for 10 min at RT with DAPI (5 μg/ml). Cell images were obtained with Zeiss Axio Observer Z1 inverted confocal microscope. DAPI, Doxorubicin and Alexa Fluor 647 fluorescence was excited at 353, 493 and 653 nm, and emission was measured at 410-490, 570-650 and 650-700 nm, respectively. The confocal microscope settings were kept the same between samples. Grey scale images were converted to pseudo-colors and merged by the software Fiji (ImageJ).[2] Contrast and brightness of all the images shown in Figure 6 were adjusted simultaneously.

MTT cell viability assay

Aliquots of $2x10^4$ of MES-SA and MES-SA Dx5 cells per well were seeded in 96-well plates. On the next day culture medium was replaced with 200 μl serum free DMEM medium with the addition of different amounts of Dd, Dd-DOX and free doxorubicin respectively. After 3 h incubation at 37 °C FBS was added to the final concentration of 10%. The cytotoxic effect was determined after 48 hours by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[3] Briefly, MES-SA and MES-SA Dx5 cells were incubated with MTT for 4 h. The formed formazan crystals were then dissolved for 60 min using 200 μl of alkaline DMSO containing 16 mM ammonia. The absorbance at 550 nm wavelength was measured using spectrometer (BioTEK Synergy HT Microplete Reader).

Dd lyophilization

Dd samples (300μg) in 20 mM HEPES buffer pH 7.5, 0.1 mM EDTA and 150 mMNaCl, were lyophilized in the presence of cryoprotectants - mannitol and sucrose, both at 0.4% final concentration. Dd samples were frozen in liquid nitrogen and dried using benchtop freeze dryer (Labconco). After lyophilization, Dd preparations were stored at indicated temperatures for 10 days and then reconstituted to the initial volume with distilled water. Additional sample has been frozen in liquid nitrogen and subsequently stored for 10 days at -20 °C as a control for freezing in the presence of cryoprotectants. Samples were then analyzed by electron

microscopy and In-cell ELISA assay, as previously described[1]. Briefly, HeLa cells ($2x10^4$cells per well) were seeded in 96-multiwell plates (Grainer) and left overnight to attach. The next day culture medium was exchanged with fresh serum free DMEM medium with addition of Dd in the amount of 4 ug per well. After incubation for 2 h at 37 °C, cells were rinsed three times with cold PBS and fixed with methanol at -20 °C for 15 min. Next cells were incubated overnight at 4 °C with PBS containing 1 % Tween-20 (PBST) and 5 % FBS, then incubated for 1h at RT with rabbit polyclonal anti-Dd antibody (1:40,000). After three washes with PBST, cells were incubated with anti-rabbit HRP-conjugated antibody (1:20,000) for 1 h at RT. After three washes with PBST, TMB substrate for HRP detection was added (Thermo Scientific). The reaction was stopped with 2 M sulfuric acid, and the absorbance was measured spectrometrically at 450 nm waveleght (BioTEK Synergy HT Microplete Reader).

Electron microscopy analysis

Dd samples were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with uranyl acetate. Micrographs were taken under low-dose conditions with a Jeol 1200 EX II microscope (Tokyo, Japan) at 100 kV and a nominal magnification of 23,000.

References

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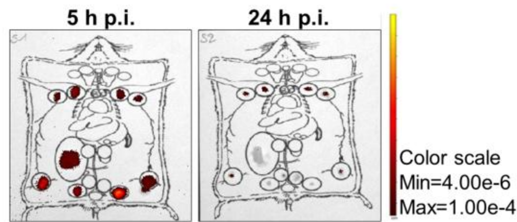
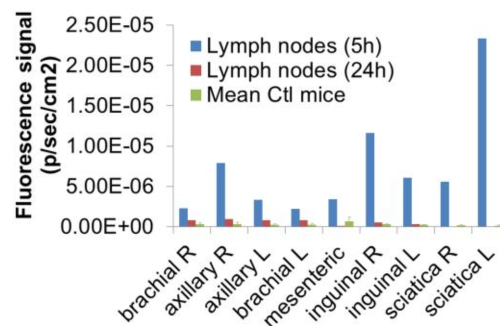
3. Wang H, Wang F, Tao X, Cheng H. Ammonia-containing dimethyl sulfoxide: An improved solvent for the dissolution of formazan crystals in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. *Analytical Biochemistry* 2012;**421**:324-6.

Supplemental Figure S1

A





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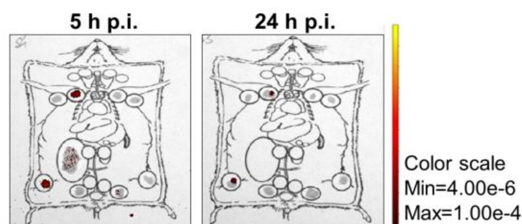

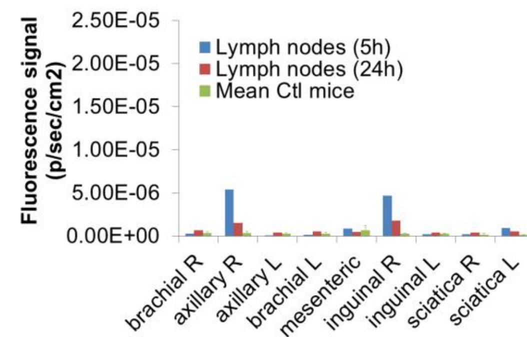

Figure S1. Dd-AF680 localization in lymph nodes. Nude mice bearing melanoma tumors were injectedA) intravenously with 33 µM (200 µl) Dd-AF680 or B) intratumorally with 8.25 µM (50 µl) Dd-AF680. Left panels: *ex vivo* fluorescence imaging of isolated lymph nodes 5 and 24 h p.i.; right panels: *ex vivo* fluorescence analysis of lymph nodes for Dd-AF680 injected and control (Ctl, non-injected) mice at indicated times post injection (p.i.).p/sec/cm^2 - number of photons per second, per square centimeter.