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A Commentary " Virus-like Particle-Mediated Intracellular Delivery of mRNA Cap Analog With *in Vivo* Activity Against Hepatocellular Carcinoma"

Zochowska M, Piguet AC, Jemielity J, Kowalska J, Szolajska E, Dufour JF and Chroboczek J*
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Commentry

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***For Correspondence**

Jadwiga Chroboczek, Therex, TIMC-IMAG, CNRS, Université Grenoble, Alpes, La Tronche, France and the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

E-mail: jadwiga.chroboczek@imag.fr

For several years I collaborate with Dr Ewa Szolajska's laboratory in my old Alma Mater, the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw. Together, we develop a unique biocompatible vector, a virus-like particle called adenoviral dodecahedron (Dd)[1]. Dodecahedric vector is formed from 12 copies of pentameric penton base, a protein responsible for the penetration of adenovirus serotype 3. This non-enveloped symmetrical VLP does not contain any genetic information, is biocompatible, biodegradable and employs a natural mechanism of endocytosis for cell entry [1]. It penetrates the plasma membrane efficiently and accesses the cytoplasm, whereby up to 300 000 particles can be seen in one cell *in vitro* **(Figure 1)** [2]. The vector is stable up to 50°C at pH 7–8 and can be conveniently stored, transported and used in different climates [3]. It might be used for direct delivery of bioactive molecules or as a vaccination platform [4]. Moreover, we know now that Dd is able to pass from cell to cell (our unpublished results).

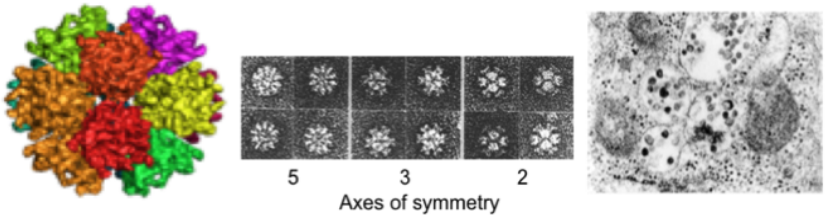

Figure 1. Dd. Left panel - cryo-electron microscopy of Dd structure at 9Å resolution [9] Middle panel - EM of purified dodecahedra. Right panel – Dd in HeLa cells. Electron microscopy was performed on thin sections, without staining. Dd is seen as black spheres in transporting vacuoles.

There is a need in research, in biological applications and in medicine for efficient biocompatible vectors, enabling passage through the plasma membrane. It is known that about 40% of newly developed drugs are rejected because of poor bioavailability. Fully defined self-assembling vectors can be built via approaches based on macromolecular chemistry and physics. These chemical vectors theoretically fulfil all the functions necessary for delivery targeted to specific diseased cells *in vivo*. However, their numerous limitations include toxicity, inability to deliver enough molecules to the cytoplasm and lack of biodegradability [5-7]. Contrary to that, virus-like particles efficiently penetrate cells by active endocytosis, are polyvalent which means that they are able to deliver multiple copies of active agents, and are fully biocompatible as they undergo proteolytic decay after cargo delivery. The only shortcoming could be the potential immune response elicited by VLPs, which might thwart repeated treatments. It is relevant however, that many proteinaceous agents are used in human medicine despite the build-up of immune response, for example asparaginase (Elspar) for acute leukemia treatment. Moreover, the penton base, a building block of Dd, is one of less immunogenic adenoviral proteins [8].

We wanted to employ the remarkable cell entry properties of Dd for drug delivery. In the first approach we covalently

attached bleomycin (BLM), an anticancer antibiotic, to Dd. Cell penetration of Dd-BLM conjugate, similarly as for free BLM, caused dsDNA breaks and induced death of transformed cells. However, an effective cytotoxic concentration of BLM delivered with Dd was 100 times lower than that of free BLM [9].

At that time we started to be interested in the role of the eukaryotic initiation translation factor 4E (eIF4E), a cap-binding protein. The eIF4E is an oncogene, with elevated expression in cancers, where it promotes tumor growth, and where a high eIF4E level correlates with poor prognosis [10,11]. The factor is known to accumulate in the cell nucleus [12]. The cytoplasmic eIF4E acts in protein synthesis, while the nuclear eIF4E is involved in transport to the cytoplasm of a subset of messenger RNAs that code for proteins required for cell proliferation. As these proteins are short living, the neoplastic cells need their constant synthesis for cycling [10,13]. During this period we started to collaborate with the team of Dr Jacek Jemielity and Dr Joanna Kowalska in the Centre of New Technologies of the University of Warsaw, specializing in chemistry of cap structure and cap analogs. They have shown that cap analogs can be used as inhibitors of excessive cap-dependent translation [14]. However, cap analogs are unable to penetrate live cells on their own. It was clear to us that they could be delivered in a form of Dd conjugates.

At that point we started looking for a clinical laboratory disposing an animal cancer model that could help in showing the potency of our delivery vector in cancer treatment. Professor Jean-Francois Dufour, a hepatologist from the Department of Clinical Research of the University of Berne in Switzerland showed plenty of courage agreeing to collaborate with us, with a goal of trying an unproven and innovative cancer treatment in an orthotopic hepatocellular carcinoma (HCC) rat model. We planned with Ewa that our common PhD student, Monika Zochowska, will prepare the material for *in vivo* studies with the help of Jacek's team in Warsaw and will use it during animal experiments with the team of Jean-Francois in Bern. The plan looked quite nice, however, for its implementation we needed money, money, money. First, we were able to get for Monika a 6-months fellowship from SCIEX (Scientific Exchange Programme between the New Member States of the EU and Switzerland). Then all collaborating laboratories pledged covering expenses: Warsaw and Grenoble for biochemistry, chemistry and molecular biology experiments and Bern for animal experiments.

Monika as an experimentalist was endowed with a clear sense of line not to be crossed and so she was quite successful. Unfortunately, her English conversation skills were non-existent, even that she was able to follow the manuals in English. So it took plenty of patience and plain kindness from Jean-Francois and in particular from Anne-Christine Piguet, a post-doctoral fellow in the University Clinics of Visceral Surgery and Medicine in Inselspital Berne, responsible for animal experiments, to work with Monika. SCIEX provided for some travel expenses for a mentor, so I visited the Bern team during Monika's stay, discussing results and trying to somewhat improve communication.

We used Dd for delivery of chemically attached cap analog and doxorubicin in an orthotopic HCC rat model. Dd-mediated delivery of these agents resulted in statistically significant inhibition of tumor growth (40%) correlated with abolished expression of two oncogenes, eIF4E and c-myc **(Figures 2 and 3)** [15]. Importantly, upon 5-week treatment the animals did not show any adverse effects such as weight loss or hyper- and hypoactivity, indicating lack of acute toxicity of a vector (or the conjugates).

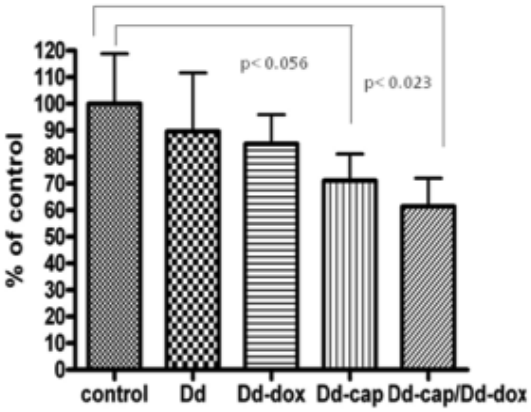

Figure 2. Groups of 6-7 rats were intraperitoneally injected once a week for 5 weeks, with Dd alone, or Dd-cap, or Dd-dox and with combination of Dd conjugates. The control treatment with free doxorubicin was omitted in order to spare the animals, as the effects of such a treatment are well known. Indeed, doxorubicin was routinely used as a single drug for advanced HCC, but has shown low efficacy, with a response rate of 15-20%.

The main problem we had with the animal experiments was the amount of material. It turned out that the amount of active conjugates we needed for animal studies was totally beyond comparison with the amount we were accustomed to need for *in vitro* studies. I am convinced that the inhibition of tumor growth **(Figure 2)** could be much more significant were we able to have more material. As you see from **Figure 3**, having not sufficient amount of our conjugates we had to use only half doses of both of them in the combination treatment.

One of auxiliary experiments gave quite interesting results. Blood (plasma), tumors and livers harvested from the treated animals were probed for the presence of the vector. Quite a pronounced reaction with anti-Dd antibody could be seen in blood

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and in tumor tissue **(Figure 4B).** As Dd runs on native agarose gel faster than its fragments [3], the appearance of Ab-reactive material retarded in comparison with the material of origin indicates that after 2 h in the animal body the Dd did not exist as a large dodecahedric entity but disintegrated yielding much smaller products. Most interestingly, in the healthy liver the amount of Ab-reactive material was negligible. The observed results suggest that Dd does not transduce the healthy liver but shows an affinity for tumors. These results prompted our present work, whereby we want to identify the Dd-interacting proteins in different fractions of normal and neoplastic cells [1]. Working with a virus-like particle that is non-infectious and does not multiply since devoid of native genetic information, we encounter problems, since the inattentive reviewers of our projects quite often think that our vector is a virus.

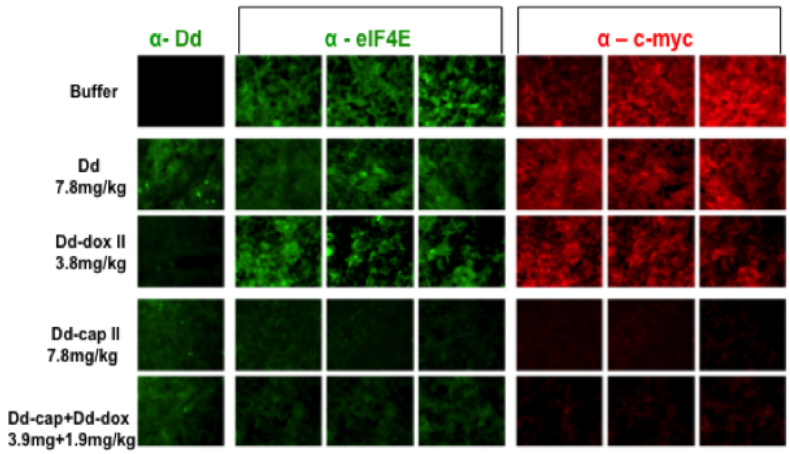

Figure 3. The immunohistochemistry of liver tumor tissue after application to animals of Dd and its conjugates was carried out on sections of HCC tumors stained for Dd (first column, in green), eIF4E (next 3 columns from different sections, in green) and c-myc (the last three columns, in red).

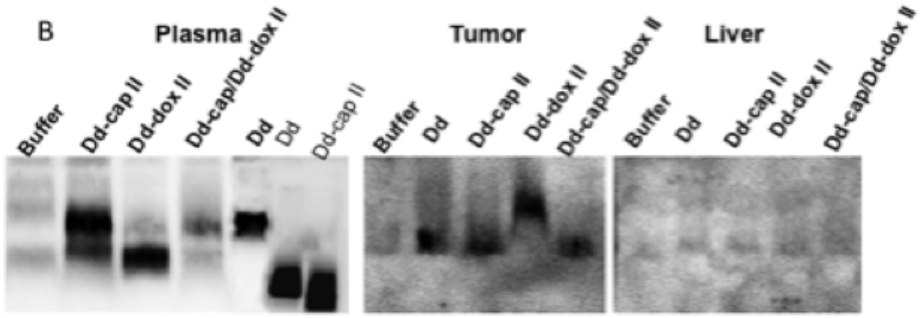

Figure 4. Dd in tissues harvested after treatment. Blood, tumors and livers were harvested from euthanized animals two hours after the last treatment. Concentrated samples derived from 50 µl plasma or tissue extracts were analyzed by non-denaturing agarose gel electrophoresis followed by Western blot performed with the anti-Dd Ab. On such gels Dd runs faster than its fragments. Control Dd and Dd-cap are shown in the last lanes of the left panel.

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