


Roberta Cavalli, Francesca Marano, Monica Argenziano, Alessandra Varese, Roberto Frairia and Maria Graziella Catalano

**Combining Drug-Loaded Nanobubbles and Extracorporeal Shock Waves for Difficult-to-Treat Cancers**

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Despite the general great improvement in cancer therapy, to date, some aggressive tumors are still without an efficient therapy. Therefore, accurate delivery of anti-cancer drugs is a very important goal in order to obtain a successful therapy and reduce systemic side effects.

Nanobubbles (NBs) are spherical core/shell vesicles filled by a gas with sizes in the nanometer order of magnitude. They have gained an increasing attention for drug delivery, because they can be versatile multifunctional carriers for the targeted release of gases, drugs and genes. Particularly, NBs can carry loaded drugs to the tumor site through the blood stream, taking advantage of the enhanced permeability and retention effect, due to the defective vascular architecture of the tumor (Fang et al. 2011). Unfortunately, vessel leakage, the absence of a functional lymphatic system and an increased extracellular matrix frictional resistance may limit drug delivery (Azzi et al. 2013, Carmeliet and Jain 2011). To overcome this problem, a better drug release to cancer tissues can be obtained by combining physical triggers (e.g. ultrasounds, US) with NBs (Gao et al. 2008, Collis et al. 2010, Cavalli et al. 2012, Cavalli et al. 2016). Indeed, US causes bubble cavitation resulting in cell sonoporation and allowing the extravasation of molecules (Collis et al. 2010).

Interestingly, polysaccharide-shelled perfloropentane (PFP)-cored NBs were developed for the delivery of anticancer agents displaying good drug encapsulation efficiency and a prolonged release kinetics. This type of formulation is referred to as “nanobubbles” for sake of simplicity but it must be said that, prior to the application of external stimuli such as US, it would be more accurate to use the term “nanodroplets” when the core is constituted of PFP, being a perfluorocarbon liquid at room temperature (boiling point 29 °C) (Cavalli et al. 2015).

Extracorporeal Shock Waves (ESWs), introduced in medicine about 40 years ago (Eisenberger et al. 1978), are short-duration (<10 μs) focused acoustic waves consisting of a compressive phase (peak pressure: 10–100 MPa) followed by a tensile phase (negative pressure). ESW therapy is widely used in urology for lithotripsy to break urinary stones (Rassweiler et al. 2011). In addition to

lithotripsy, more recently, ESWs have been successfully used for the treatment of several musculoskeletal diseases (Wang et al. 2012). ESWs can be focused with high precision also in depth and they determine permeabilization of plasma membranes (Frairia et al. 2003, Catalano et al. 2007), as a consequence of cavitation occurring when shockwaves propagate into fluids. As result of induced cell permeability, ESWs increase the cytotoxic effects of different anti-cancer drugs (Frairia et al. 2003, Palmero et al. 2006, Catalano et al. 2007, Canaparo et al. 2008). Notably, ESWs differ from US as they have 1000 times higher peak pressure (Wang et al. 2012), and no heating effects. The absence of heating effect is an advantage for *in vivo* application since temperature elevation is difficult to control spatially and temporally, especially in large tumors with heterogeneous vascularization (Diederich et al. 1999). All these features make ESWs an ideal tool to be used in combination with drug-loaded NBs. Indeed, drug-loaded NBs can be injected intravenously and, when they have reached the tumor, ESWs can be applied directly at the tumor site. At this level, in fact, ESW-induced cavitation might determine perturbation of the NBs in addition to transient permeabilization of both plasma and nuclear membranes, resulting in the drug release and penetration into the cells.

The effects of combining chemotherapeutic drug-loaded NBs and ESWs have been recently investigated into two different types of aggressive cancers; e.g., anaplastic thyroid cancer (ATC) and castration resistant prostate cancer (CRPC).

In ATC, we obtained both *in vitro* and *in vivo* data. *In vitro*, the combined treatment with doxorubicin-loaded glycol chitosan NBs and ESWs enhanced cytotoxicity of doxorubicin in two different ATC cells lines (CAL-62 and 8305C), decreasing the drug growth inhibition fifty ($GI_{50}$) of 40% in CAL-62 and 46% in 8305C cells, respectively (Marano et al. 2016). The reduction of the $GI_{50}$ is notable, since the use of lower doses of doxorubicin is essential to reduce its side effects. In a xenograft *in vivo* model, after 21 days, the combined treatment determined the greatest drug accumulation in tumors with consequent reduction of tumor volume and weight, and an extension of the tumor doubling time. Mechanistically, the treatment induced tumor apoptosis and decreased

cell proliferation. Finally, loading doxorubicin into NBs avoided and prevented heart damage, the most frequent side effect of the free drug that causes heart fibrosis and oxidative stress (Marano et al. 2017).

In CRPC, Marano et al. (2016) reported that the combined treatment with either paclitaxel- or docetaxel-loaded NBs and ESWs enhanced the cytotoxicity of both taxanes in two different cell lines (PC3 and DU145), resulting in paclitaxel $GI_{50}$ reduction of about 55% and in docetaxel $GI_{50}$ reduction of about 45%, respectively. Moreover, the proposed combination therapy was effective also on cell migration. Paclitaxel-loaded NBs and ESWs reduced cell migration by more than 85% with respect to paclitaxel alone; whereas docetaxel-loaded NBs and ESWs reduced cell migration by more than 82 % with respect to docetaxel alone.

Given the promising results of these preclinical studies, combining drug-loaded NBs and ESWs should be taken into account also for other aggressive tumors in which chemotherapy remains the first option. The next step will be conducting clinical trials with the hope of improving cancer treatment as well as patient quality of life.

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