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Possible Applications of Polymer Nitric Oxide Donors in Treatment of Experimental Murine Tumors

Možnosti využití polymerních donorů oxidu dusnatého
pro léčbu myších experimentálních nádorů

Diploma Thesis

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Prohlášení:

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Veronika Horková

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Abstract

Polymer-based drug delivery systems represent one of the promising strategies for successful tumor treatment. Conjugation of a low-molecular-weight drug to a synthetic polymer carrier enables targeted drug delivery to tumor tissue/cells and limited systemic toxicity of the drug. The conjugates show extended circulation time, and preferentially accumulate in tumor tissue due to the Enhanced Permeability and Retention (EPR) effect. The EPR effect depends on a structural anomaly in tumor neovasculature, and vasodilators were shown to enhance the EPR effect via an increase of blood supply in the tumor. Polymer drug carriers based on water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) benefit from variable architecture, drug loading and controlled release. HPMA-based conjugates with cancerostatics have already proved high anti-tumor activity, inducing complete tumor regression followed by resistance to a second tumor challenge in experimental murine models.

Three HPMA-based conjugates with organic nitrates (labeled 1, 2, and 3) were prepared as polymer donors of nitric oxide (NO) with the aim to intensify the EPR effect, thereby enhancing accumulation of co-administered macromolecular cancerostatics in the tumor.

In this study, the conjugates were non-toxic to cancer cells and did not potentiate the cytostatic effect of cancerostatics *in vitro*. In mice, conjugates 1 and 2 showed considerably low systemic toxicity. Conjugate 1 enhanced the EPR effect, as it significantly increased doxorubicin (Dox) content in the EL4 lymphoma tumors following Dox delivery by high-molecular-weight polymer carrier, in comparison with the administration of the Dox-conjugate only. In the EL4 lymphoma model, treatment with the star HPMA-Dox conjugate together with conjugate 1 or 2 resulted in a significantly improved outcome. However, conjugate 1 did not improve the treatment of difficult-to-treat 4T1 breast carcinoma. Conjugate 1 also showed a certain capacity in regulation of anti-tumor immune response, as it could modulate expression of CD40 on murine bone marrow-derived dendritic cells *in vitro*.

Keywords: *N*-(2-hydroxypropyl)methacrylamide, HPMA, Targeted Tumor Therapy, Polymer Drug Delivery Systems, EPR Effect, Solid Tumor, Organic Nitrates, Nitric Oxide, Nitric Oxide Donors

Abstrakt

Léčba založená na využití polymerních systémů pro cílenou terapii představuje jednu ze slibných strategií úspěšné léčby nádorů. Navázání nízkomolekulárních léčiv na syntetický polymerní nosič umožňuje cílený transport léčiv do nádorové tkáně/buněk a omezení systémové toxicity léčiva. Tyto konjugáty mají prodlouženou dobu cirkulace a díky efektu zvýšené permeability a retence (EPR efektu) se přednostně akumulují v nádorové tkáni. EPR efekt je založen na strukturní anomálii neovaskulatury nádoru a použití vasodilatačních látek zesílilo EPR efekt zvýšením krevního zásobení tumoru. Polymerní nosiče léčiv na bázi vodorozpustného *N*-(2-hydroxypropyl)methakrylamidu (HPMA) mohou využívat výhod různé architektury, obsahu léčiva a jeho kontrolovatelného uvolňování. Konjugáty na bázi HPMA s kancerostatiky již prokázaly vysokou protinádorovou aktivitu, indukci úplné regrese nádoru, která je v experimentálních myších modelech následována vývojem rezistence vůči další transplantaci nádorových buněk.

S cílem zesílení EPR efektu vedoucího ke zvýšené akumulaci makromolekulárních léčiv v nádoru byly připraveny tři konjugáty s organickými nitráty založené na HPMA (označené 1, 2 a 3) jako polymerní donory oxidu dusnatého (NO).

V předložené studii tyto polymerní NO donory nevykazovaly toxicitu vůči nádorovým buňkám a nezesilovaly cytostatický efekt cancerostatik *in vitro*. Konjugáty 1 a 2 ukázaly výrazně nízkou systémovou toxicitu u myší. Konjugát 1 zesiloval EPR efekt, protože významně zvyšoval obsah doxorubicinu (Dox) v nádorech EL4 lymfomu, když byl Dox aplikován ve vazbě na vysokomolekulární polymerní nosič v porovnání s aplikací samotného konjugátu s Dox. U modelu lymfomu EL4 měla léčba hvězdicovitým konjugátem na bázi HPMA s Dox v kombinaci s konjugátem 1 nebo 2 významně lepší výsledek. Konjugát 1 však nezvýšil účinnost léčby u obtížně léčitelného myšího karcinomu mléčné žlázy 4T1. Konjugát 1 také prokázal určitou schopnost regulace protinádorové imunitní reakce, protože ovlivňoval expresi CD40 molekul na myších dendritických buňkách z kostní dřeně *in vitro*.

Klíčová slova: *N*-(2-hydroxypropyl)methakrylamid, HPMA, cílená léčba nádorů, polymerní systémy transportu léčiv, EPR efekt, solidní tumor, organické nitráty, oxid dusnatý, donory oxidu dusnatého

Contents

Abbreviations

2-ME	2-mercaptoethanol
ABC	ATP-binding cassette
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BM-DC	Bone-marrow derived dendritic cells
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CTL	Cytotoxic T lymphocyte
Da	Dalton
DC	Dendritic cell
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
Dox	Doxorubicin
Dtx	Docetaxel
eNOS	Endothelial nitric oxide synthase
EPR	Enhanced permeability and retention
EtOH	Ethyl alcohol
FACS	Fluorescence-activated cell sorting
FA-OMe	Fluoresceine amine methyl ester
FCS	Fetal calf serum
Glc	Glucose
Glu	Glutamine
GTP	Guanosine triphosphate
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIF-1-α	Hypoxia-inducible factor 1-α
HMW	High-molecular-weight
HPLC	High-performance liquid chromatography
HPMA	*N*-(2-Hydroxypropyl) methacrylamide
IL	Interleukin
IMC	Institute of Macromolecular Chemistry
iNOS	Inducible nitric oxide synthase
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LMW	Low-molecular-weight
LPS	Lipopolysaccharide

M	Molar concentration (mole/liter)
MD-DC	Monocyte-derived dendritic cells
MDR	Multi-drug resistance
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MTT	Thiazolyl blue tetrazolium bromide
M_w	Molecular weight
M_w/M_n	Polydispersity index
NaPyr	Sodium pyruvate
NG	Nitroglycerin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate-buffered saline
Ptx	Paclitaxel
RFU	Relative fluorescence unit
rGM-CSF	Recombinant granulocyte macrophage colony-stimulating factor
R_h	Hydrodynamic radius
RNS	Reactive nitrogen species
RPMI	Roswell Park Memorial Institute Medium
S.D.	Standard deviation
SNP	Sodium nitroprusside
VEGF	Vascular endothelial growth factor

1. Introduction

Although there have been vast improvements in cancer therapy in the last few decades, surgery, chemotherapy and radiation are still major approaches in cancer treatment. Most of these approaches do not discriminate between tumor and healthy tissues, including tumor vs. immune cells. Although immunotherapy has emerged as a novel method, it still comes with many limitations and side-effects and its combination with other modalities is necessary. The targeted therapy of tumors has developed in the last few decades, but tumor diversity is vast and finding universal or even tumor type-specific targets for actively-targeted therapy has been mostly unsuccessful.

Nowadays, one of the most promising approaches is to overcome systemic toxicity by binding the low-molecular-weight (LMW) drugs to nano-sized carriers, creating devices that can preferentially pass through the endothelium in a tumor. It significantly reduces side-effects and appears to be a more or less universal mechanism for all the vascularized solid tumors. The concept of polymer-based targeting of tumor therapy was coined by Ringsdorf approximately 40 years ago (Ringsdorf, 1975) and since then the study of the concept has expanded.

1.1 Polymer-based Tumor Therapy

In 1986, Matsumura and Maeda (Matsumura & Maeda, 1986) described the Enhanced Permeability and Retention (EPR) effect in murine solid tumors. Later, it was also proved as valid in human solid tumors (Gabizon *et al.*, 1994). The EPR effect is present due to leaky vasculature in tumors and this defect of endothelium allows for macromolecules larger than approximately 40 kDa to preferentially extravasate into tumor tissue (Noguchi *et al.*, 1998) (see Figure 1.1). A lack of lymphatic drainage in a tumor microenvironment allows the retention of macromolecules and nano-devices in the tumor. It has been detected that molecules with sizes even up to 1 µm can pass through the endothelial barrier in tumor capillaries. This was demonstrated by detection of bacterial accumulation in tumors (Zhao *et al.*, 2005).

High-molecular-weight (HMW) polymer carriers can be prepared from substances naturally occurring in the body or synthesized chemically. They can be passively targeted to the tumor due to the EPR effect or actively targeted to the specific structures/receptors in the tumor microenvironment. There are many forms and sizes in which these nano-sized devices can be prepared – dendrimers, micelles, liposomes,

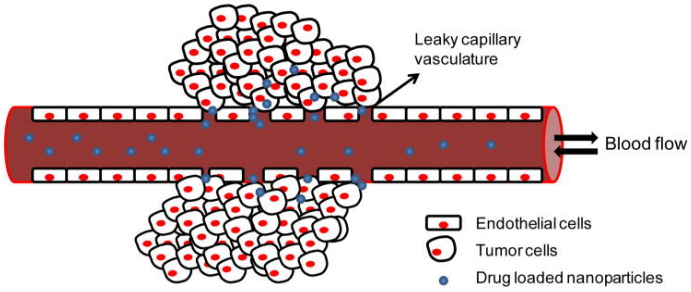

Figure 1.1: **EPR effect demonstrated by passive nanocarrier targeting.** Edited from Biswas & Torchilin (2014)

nanospheres, hydrogels, soluble polymer systems of various carrier architecture etc. They can "carry" the active molecules through a variety of physical bonds, either covalently bound or encapsulated by electrostatic or hydrostatic interactions.

One of the main requirements for the nano-sized devices is biocompatibility, which enables various delivery routes to a specific tissue or cell compartments. Most of these systems allow longer persistence in circulation without any undesirable toxicity or immune reaction towards the carrier. The transport systems should eventually also be degradable, allowing for their ultimate elimination from the organism.

The main advantage of polymer-based tumor therapy is the elimination of side-effects. The therapy is mostly administered intravenously and nano-sized devices do not penetrate to the healthy tissues, but they persist in circulation. The active molecules bound to the nano-sized carriers can potentially manifest some effect within the circulation. Avoiding such mechanisms can be achieved by intratumoral administration of the nanoparticles (Lammers *et al.*, 2006). However, any physical manipulation within the tumor is considered to be risky in the clinical conditions in some types of cancer (Shyamala *et al.*, 2014). Moreover, the intratumoral injection is physically applicable only in tumors which are easily detectable and accessible. In patients with progressive metastasis, such application would not be feasible.

Polymer-based tumor therapy brings a vast amount of possibilities for utilization and is therefore one of the most promising approaches in tumor treatment.

1.1.1 HPMA-based Therapy

In the early 1970s, professor Kopeček and his colleagues were working on the synthesis of biocompatible polymers, which could be used as plasma expanders. The research resulted in the synthesis of *N*-(2-Hydroxypropyl) methacrylamide (HPMA) polymers

(Kopecek *et al.*, 1973). These polymers were eventually used as carriers of LMW chemotherapy for targeted tumor treatment (Kopecek *et al.*, 1991). The application of HPMA-based copolymers with chemotherapeutics for treatment of a wide variety of experimental murine tumor models showed a significantly improved treatment outcome when compared to LMW drugs (Rihova *et al.*, 1997; Minko *et al.*, 2000; Rihova *et al.*, 2003).

The HPMA-based polymer drug carriers have many advantages compared to other macromolecular delivery systems. They are hydrophilic, non-toxic, non-immunogenic (Rihova *et al.*, 1989) and have multiple functional groups for binding the active molecules. Combinations of these characteristics provide the best template for the administration of LMW substances, or molecules that are hydrophobic and therefore cannot be administered intravenously in the form of aqueous solutions.

Moreover, the HPMA-based copolymers allow for assembly into various forms by several types of linkers. Firstly, linear copolymers were created (Kopecek *et al.*, 1991), moving e.g. to branched structures (Dvorak *et al.*, 1999), star-like structures with dendrimer core (Etrych *et al.*, 2011) or micelles (Chytil *et al.*, 2008), further increasing the number of potential binding sites for drugs and increasing M_w and the hydrodynamic radius of the carrier.

HPMA-based copolymers may carry the active drug using several types of linkers. Various linkers have been tested; the best ones that were preferentially explored in conjugates were enzymatically degradable (Ulbrich *et al.*, 1980) or pH-sensitive (Etrych *et al.*, 2001). Among pH-sensitive spacers, the cis-aconityl spacer and the hydrazone bond were widely studied. Now, the HPMA copolymers designed and synthesized for our laboratory explore the pH-sensitive hydrazone bond preferentially, as it showed excellent performance with different carrier structures and LMW drugs.

Not only the size of these copolymers, but also the linker used for the attachment of the drug is an important element for cellular and subcellular localization of the HPMA-based polymer drug in the target cells (Hovorka *et al.*, 2006). Moreover, a recent study showed that linear and star-like structure of HPMA-based copolymers with hydrazone bound anthracycline Doxorubicin (Dox) exhibit different pharmacokinetics and different levels of toxicity (Tomalova *et al.*, 2015), not necessarily in favor of larger copolymers.

The HPMA-based copolymers have been mainly intended for cancer treatment. Not only solid tumors can be targeted by this type of treatment. They have been also used in treatment of experimental leukemia. Even when there is no tumor for accumulation, the enhanced retention in circulation allows for better efficiency of chemotherapy for

leukemia (Tomalova *et al.*, 2015).

Various anti-tumor drugs have been bound to the HPMA copolymers. The most widely used ones are copolymers with anthracyclines, mainly with Dox, however, copolymers with taxanes such as Docetaxel (Dtx) (Etrych *et al.*, 2015), Paclitaxel (Ptx) (Etrych *et al.*, 2010) and platinates such as cisplatin (Gianasi *et al.*, 1999) or oxaliplatin (Nowotnik & Cvitkovic, 2009) have been tested in solid tumor treatment. The first HPMA-based copolymer with Dox (PK1) entered clinical trial in 1994 (Vasey *et al.*, 1999), and was followed by other copolymers e.g. with Ptx (Meerum Terwogt *et al.*, 2001) or platinates (Rademaker-Lakhai *et al.*, 2004; Nowotnik & Cvitkovic, 2009). These trials helped to further understand and develop the HPMA-based therapy and improve its safety and effectiveness, but none of the HPMA-copolymers have been approved for clinical use. There have also been further attempts at introducing HPMA-based copolymers into clinical practice, such as a clinical trial for the treatment of breast carcinoma in Prague (Rihova *et al.*, 2003). HPMA-based therapy in clinical trials is reviewed in Rihova & Kubackova (2003); Rihova (2009).

When conventional chemotherapy is used in patients, tumors often develop multi-drug resistance (MDR), mainly mediated by upregulation of ABC transporter expression. These transporters pump the drug out of the cytoplasm of the cancer cells. Long-term exposure to the LMW drugs enables the selection of drug-resistant clones. However, long-term exposure to HPMA-based copolymers with Dox did not lead to the induction of MDR *in vitro* (Minko *et al.*, 1999). The HPMA-based copolymers carrying both chemotherapy and inhibitors of the ABC transporters have been shown as an example of a successful strategy to overcome existing MDR (Subr *et al.*, 2014). Overcoming MDR is another reason and mechanism that needs to be taken into consideration during the development of strategies for the augmentation of chemotherapy treatment.

1.2 Nitric Oxide

Nitric oxide (NO), a small molecule with a size of 115 pm and a biological half-life of approximately 3-6 seconds, depending on the amount of oxygen present (Kelm *et al.*, 1991), it has numerous effects in the organism. It is an important element of signaling, post-translational regulation, vascular function, immune responses and other mechanisms. The variety of effects, for example vasodilatory effect provides potential for this molecule to be used for treatment, e.g. of cardiovascular diseases. However, as NO has such a short half-life, molecules from which NO can be generated in the

organism have to be used. Fortunately, there are plenty of such molecules, both natural and synthetic. However, their metabolization is also very fast and often unexplained. Vast amounts of NO in an organism can lead to rapid hypotension, resulting in organ damage and possibly death. NO is also toxic to cells if present in higher concentrations (especially to hepatocytes).

NO is generated within our body from L-arginine. NO synthase (NOS) is an enzyme which can transform L-arginine to NO and citrulline. There are three types of this enzyme, expressed within several various types of tissues - neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). nNOS is expressed in neurons, eNOS is expressed in endothelial cells and iNOS can be expressed in almost all cell types, typically in cells of myeloid origin.

Although nNOS is expressed in neurons, all three forms of NOS can be expressed in the brain. NO appears to be important for brain functioning, as inhibitors of NOS have shown to impair learning and memory skills, while a supplementation of L-arginine showed a reverse effect (Paul & Ekambaram, 2011). An impairment of NO synthesis was connected with neurodegenerative diseases such as Alzheimer's disease (Toda & Okamura, 2012).

eNOS is important in the regulation of blood flow in the whole body. When NO is synthesized by eNOS, it diffuses into the smooth muscle cells in the blood vessel walls, interacts with guanylate cyclase, allowing for the transformation of GTP to cGMP, which then activates the protein kinase G that phosphorylates proteins responsible for the regulation of the calcium concentration. This phosphorylation results in hyperpolarization and further modification of the cytoskeleton which causes smooth muscle relaxation (see Figure 1.2A) and therefore vasodilation.

NO donors have been used for the treatment of cardiac diseases for more than a century now. The first such molecule was amyl nitrite, used for the treatment of angina pectoris since 1867. Later, it was replaced by an organic nitrate - glycerol trinitrate (nitroglycerin – NG), which is still currently used for the treatment of angina pectoris. Vasodilatory effects of NO donors are used not only for the treatment of patients, NO is widely used as a food supplement in the fitness industry.

Another form of NOS synthase is iNOS, that may be expressed in a variety of cell types. Its important function is in macrophages and other cells of innate immunity, which produce NO to fight against infections. As NO is a radical, it manifests cytotoxic activity towards a variety of pathogens. During immune response to inflammation, iNOS synthesis is stimulated by cytokine signalization (see Figure 1.2B). NO that is metabolized by iNOS from L-arginine either operates directly or reacts with oxygen,

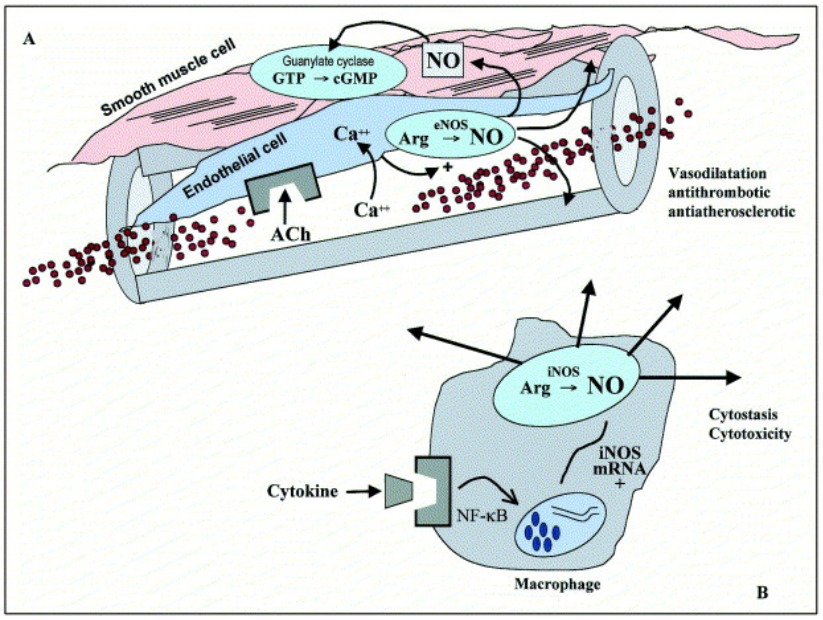

Figure 1.2: **Effects of endothelial and inducible NO synthases.** Adopted from Witte & Barbul (2002)

creating reactive nitrogen species (RNS). In both forms, NO may interact with metal centers, inhibiting cell respiration in bacteria (Brown, 2001). RNS may also affect bacterial DNA stability (Salgo *et al.*, 1995; Wink *et al.*, 1991) and the nitrosative stress may interfere with other pathways.

The main radicals formed from NO which cause such damage to bacteria are nitrosonium (NO^+) and peroxynitrite ($ONOO^-$). Bacteria develop protective mechanisms to eliminate the effect of such molecules by converting NO to nitrates via hemoglobin molecules (Crawford & Goldberg, 1998). Even if many bacteria can induce such resistance, nitrosative stress is still a powerful agent in innate immune response to infection. However, if the condition of sepsis occurs, the elevated level of NO can cause hypotension resulting in tissue damage (Titheradge, 1999).

The anti-bacterial effect of NO has been translated into the idea of coating materials by NO donors, in order to interfere with the formation of biofilm, which could compromise any manipulation with instruments, for example during some examinations or surgeries (Zhang *et al.*, 2002) or during implant application (Nablo *et al.*, 2005). This approach seems to be quite successful in the reduction of both Gramm positive and negative bacteria (Cai *et al.*, 2012).

Not only foreign organisms, but also damaged self tissues can trigger the immune response. Similarly, NO is important in this process. It is also synthesized by iNOS and L-arginine has been shown to be critical in wound healing (Schaffer *et al.*, 1996).

However, iNOS can compete with arginase for L-arginine in some situations, such as chronic inflammation or tissue repair (Das *et al.*, 2010). Arginase breaks down the L-arginine, what results in the formation of ornithine that serves as a precursor for proline formation that is important in collagen synthesis (Albina *et al.*, 1993). Furthermore, NO influences the activity of protein kinase C, which is important in collagen metabolism in fibroblasts (Craven *et al.*, 1997). NO has also been argued to have a role in metalloproteinase activity (Ridnour *et al.*, 2007) and vascular formation (Matsunaga *et al.*, 2002). These effects of NO clearly pinpoint its importance in tissue repair and wound healing.

1.2.1 NO and Cancer

The role of NO in various aspects of malignant transformation and cancer progression have been studied in the last few decades. There are many mechanisms where NO can act as pro-tumorigenic and plenty of those where an anti-tumorigenic effect is observed.

One of the claimed pro-tumorigenic effects is the involvement of NO in Wnt signaling. The iNOS enzyme contributes to the production of RNS, which are critical for inflammation-associated carcinogenesis. It has been shown that RNS are capable of mutating β-catenin gene, which leads to the accumulation of β-catenin that upregulates Wnt signalization (Du *et al.*, 2013). This signal upregulation has been immensely associated with carcinogenesis. A direct link between RNS and carcinogenesis has been shown e.g. as RNS influence inflammation-based transformations of benign murine fibrosarcoma to metastatic malignant forms (Okada *et al.*, 2006).

On the other hand, RNS have been also shown to induce apoptosis in tumors at higher concentrations (Hirst & Robson, 2010). Moreover, NO activates the tumor-suppressor p53 (Wang *et al.*, 2002), but in NO overproduction, p53 serves as a negative regulator of iNOS synthase expression (Ambs *et al.*, 1998). The interrelation between these two molecules has been mentioned in several studies but remains unresolved.

It has been shown that defects in NO formation are also important in vascular formation and maintenance in tumors. The administration of NO donors normalized the vasculature in tumors, allowing for better oxygenation and therefore improvement of chemo/radio-therapy that is not efficient in hypoxic conditions (Kashiwagi *et al.*, 2008). NO itself also appears to reduce hypoxia-induced resistance in breast cancer cells (Frederiksen *et al.*, 2007). The direct sensitization of cells by NO to tumor treatment using radiotherapy or cisplatin has been detected (Ning *et al.*, 2014).

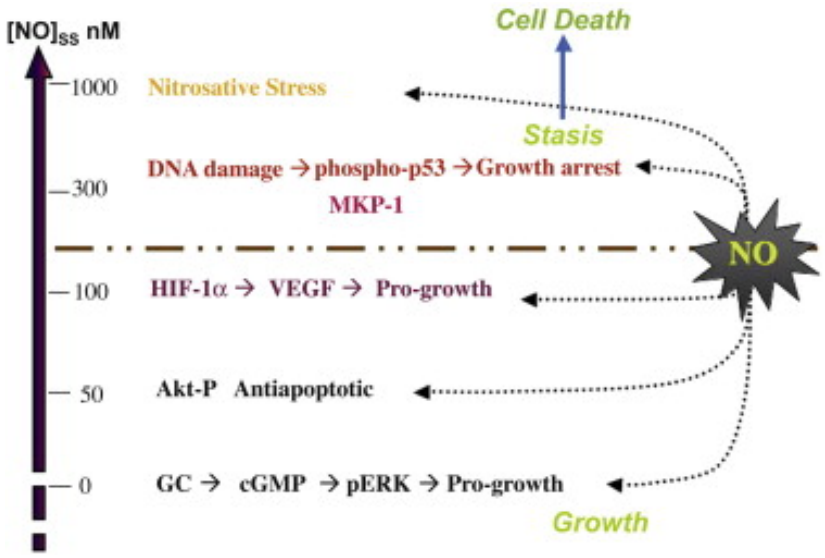

Figure 1.3: **Threshold of NO function in cancer.** Adapted from Ridnour *et al.* (2008)

The role of NO in cancer appears to be dose-dependent. However, this can differ depending on the model used for study. In 2008, Wink and his group reviewed data on the mechanism of NO in cancer and came up with the idea of a threshold between the pro- and anti-tumorigenic effect of NO concentration at a range between 100 and 300 nM (Ridnour *et al.*, 2008). Concentrations lower than 100 nM were considered to enhance tumor growth, whereas concentrations higher than 300 nM contributed to the death of tumor cells (see Figure 1.3). Since then, however, a vast amount of data has emerged, further explaining that not only NO concentration, but many more conditions in the organism can influence the function of NO in cancer.

In a recent review, the overall concept of high vs. low concentrations was not negated (see Figure 1.4), however, the context of cellular microenvironment and the differences between cancer cell origin were stressed to be very important (Witte & Barbul, 2002). For example, iNOS deletion in two tumor models led to a different outcome – in the murine ovarian sarcoma, metastatic tumor growth was increased, whereas in murine melanoma metastatic growth was reduced (Shi *et al.*, 2000). Tissue origin of iNOS may also play role in the metastatic activity of a tumor. Whereas iNOS in tumor cells decreases metastatic potential in murine breast carcinoma, iNOS expression in stromal cells increases metastatic growth (Gauthier *et al.*, 2004).

Apart from the review by Witte & Barbull, in recent years, a quite high number of reviews on NO and its function in cancer and its treatment has been released (Cheng *et al.*, 2014; Vasudevan & Thomas, 2014; Chang *et al.*, 2015; Bonavida & Garban, 2015). All authors call for a better understanding of mechanisms involving NO and

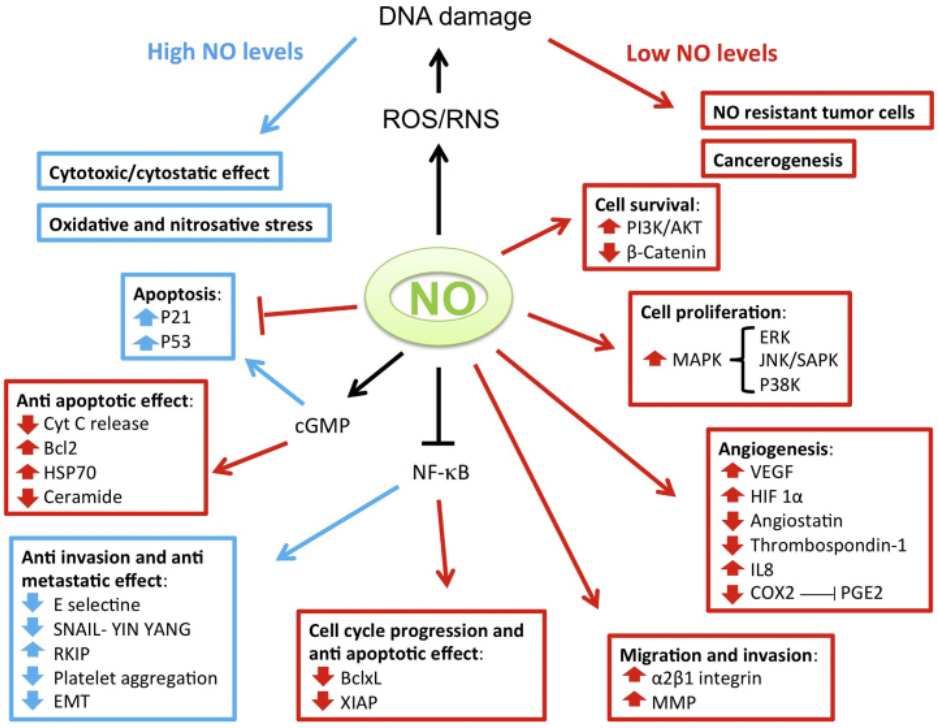

Figure 1.4: **Mechanisms involved in NO function in cancer.** Adapted from Vanini *et al.* (2015)

therefore a more controlled delivery of NO donors.

1.2.2 NO and Adaptive Immunity

The diverse role of NO in adaptive immune response has also been disputed. It has been suggested that small levels of NO contribute to the differentiation of Th1 cells, whereas larger concentration of NO are toxic to the T cells (Niedbala *et al.*, 1999). NO has also been shown to mediate T cell suppression by mesenchymal stem cells (Sato *et al.*, 2007). The expression of iNOS has an important role in the protection of plasma cells against cell death (Saini *et al.*, 2014).

In a tumor microenvironment, dendritic cells (DCs) are often induced towards a tolerogenic phenotype. The effect of NO on murine and human dendritic cells and their antigen-presenting function in tumors has been studied. When DETA-NO (LMW NO donor) was used for pretreatment of murine BM-DC, their resistance to tumor-induced apoptosis increased significantly and they were able to induce T-cell proliferation and reduce the growth of murine melanoma tumor (Perrotta *et al.*, 2004). Human monocyte-derived DCs (MD-DC) generated from peripheral blood that were treated by DETA-NO did not influence T-cell proliferation. The combination of

treatment of MD-DC by IFN-γ and DETA-NO induced a much higher proliferation of T cells than IFN-γ stimulation alone (Paolucci *et al.*, 2003).

When human MD-DC stimulated to maturation by lipopolysaccharide (LPS) were treated by another NO donor S-nitrosoglutathione (GSNO), their capacity to activate T cells decreased and it led to a differentiation towards the Th1 phenotype (Corinti *et al.*, 2003). Treatment of murine BM-DC by another NO donor chloroquinone did not induce T-cell proliferation (Thome *et al.*, 2014). When the $CD4^+$ T cells from murine spleen and lymph nodes were directly stimulated by DETA-NO, they developed the phenotype of regulatory T cells (Tregs) (Niedbala *et al.*, 2006). These NO-induced Tregs were able to suppress the differentiation of T cells towards the Th17 phenotype, but not towards the Th1 phenotype (Niedbala *et al.*, 2013).

In 1955, Thomlison and Gray observed that hypoxia develops in solid tumors (Thomlinson & Gray, 1955). The role of NO in the regulation of hypoxia-inducible factor 1α (HIF-1-α) has been shown to be important (Agani *et al.*, 2002). The NO-mediated downregulation of HIF-1-α has been shown to result in diminished PD-L1 expression on cancer cells, therefore limiting the resistance of the cancer cells to CTL-mediated lysis (Barsoum *et al.*, 2014).

The role of NO in adaptive immune response appears to be very complex and mostly unclear. It appears to differ in mice and in human cells, it probably differs between various cell types and subtypes and it is, most likely, the sum of all the effects that leads to the overall outcome of NO regulation, depending on the microenvironmental context.

1.3 Augmentation of EPR Effect

As much as polymer-based treatment with chemotherapy is successful, there are still experimental tumors that are less responsive to therapy, including that using polymer therapies. Any improvement of the treatment is therefore desirable, increasing the EPR effect being one of the potential ways. As the accumulation of polymer drugs is facilitated through the blood flow in the tumor, stimulation of the circulatory system opens some possibilities. In the early 1990s, an increase in blood flow induced by slow injection of angiotensin II into circulation was used to improve the accumulation of macromolecular drugs in tumor models (Li *et al.*, 1993). Since the vasculature in tumors has defective angiotensin II receptors, blood flow was only increased in a tumor microenvironment (Suzuki *et al.*, 1981).

Later it was demonstrated that the EPR effect is dependent on NO and can be inhibited using an NO scavenger (Wu *et al.*, 1998). The analogy of hypoxia in a tumor and cardiac arrest led professor Maeda to use organic nitrates, which had been used for more than century in cardiac diseases, in order to enhance the EPR effect. The application of NG ointment directly on a tumor and its surroundings caused a higher accumulation of polymer drugs (Seki *et al.*, 2009). Using NG, a higher partial pressure of oxygen present in a tumor induces the down-regulation of VEGF and expression (Yasuda *et al.*, 2006), which may lead to the inhibition of neoangiogenesis in the tumor.

There are plenty of LMW donors of NO. They include the already mentioned NG, isosorbide mononitrate, sodium nitroprusside (SNP), dizaeniumdiolates, S-nitrosothiols and many more. However, these LMW NO donors are processed to NO within the organism in a very short time. Therefore, the delivery of these substances is problematic, often used in the form of inhalation (nonselective location) (Yurtseven *et al.*, 2003), dermal creams or patches (Seki *et al.*, 2009). Some of them have shown enhanced persistence in circulation, e.g. DETA-NONOate has a biological half-life of approximately 20 hours.

The LMW NO donors exert variable vasodilation potency and have no tumor specificity in general - only a small fraction can be utilized for EPR effect augmentation. There are many disadvantages of their usage, such as rapid blood clearance, short term action and possibly lethal side-effects at higher dosages.

The increase of blood flow does not have to be caused only by NO donors, local hyperthermia has shown to improve nanoparticle extravasation to tumors (Li *et al.*, 2013).

1.3.1 Augmentation of EPR Using HMW Delivery Systems

As the LMW NO donors are problematic in terms of dosage and administration, their attachment to nanosized carriers can provide a solution. The utilization of NO donors can have a high variability and their attachment to polymer carriers or nanocarriers has been the focus of several research groups for variable applications in the last few years (Cai *et al.*, 2012; Zhang *et al.*, 2002; Duong *et al.*, 2014; Hasan *et al.*, 2015; Pena *et al.*, 2016). Using HMW (nano-sized) delivery systems for the enhancement of the EPR effect and therefore increasing the treatment outcome by HMW drugs has been emerging only recently.

Firstly, liposomes were used as carriers of LMW donor PYRRO/NO and their accumulation in lysosomes and higher toxicity towards ovarian cancer cells was deter-

mined *in vitro* (Stevens *et al.*, 2010). The first polymer-bound NO donor that was administered *in vivo* was JS-K and its analogues bound to the sugar backbone. Their s.c. administration next to the xentotransplanted human head and neck squamous carcinoma in nude-mice led to a significant decrease in tumor progression and a prolonged survival time (Duan *et al.*, 2012).

Furthermore, the encapsulation of NO in gaseous form in echogenic liposomes reached a dose-dependent cytotoxic effect of NO towards breast carcinoma cells (Lee *et al.*, 2014). Most recently, a group in Japan introduced the S-nitrosated human serum albumin dimer for the enhancement of the EPR effect in a murine melanoma model (Kinoshita *et al.*, 2015). This treatment increased the accumulation of polymer-based drugs in tumors and led to a decrease in tumor progression and better survival of mice.

2. Thesis Aims

This thesis is part of a project studying the application of polymer NO donors for the intensification of the anti-tumor effect of cytostatic drugs. The working hypothesis is that the HPMA-based polymer NO donors would be able to accumulate in the solid tumor tissue due to the EPR effect and release NO inside the tumor cells or in the tumor microenvironment, whilst not releasing NO spontaneously into circulation. High accumulation of a HMW polymer NO donor in the tumor due to molecular weight and hydrodynamic radius would lead to the biotransformation of the polymer-bound organic nitrates to NO, preferentially in the tumor microenvironment.

As a result, vasodilation specific to the tumor vessels would occur, increasing blood flow in the tumor and potentially also amplifying the leakiness of the tumor vessels. This would lead to the intensification of the EPR effect and therefore a higher accumulation of cytostatic drugs in the tumor, specifically of those delivered by a HMW polymer carrier.

The specific aims of this thesis are:

- to test whether polymer NO donors are capable of chemosensitization of murine tumor cells towards treatment with cytostatic drugs,
- to demonstrate higher accumulation of HPMA copolymers with cytostatic drugs, when using polymer NO donors in tumor models of murine breast carcinoma and T-cell lymphoma,
- to explore the effect of polymer NO donors on the treatment of experimental murine tumors with high-molecular-weight cytostatic drugs, and
- to investigate the effect of polymer NO donors on the maturation and stimulation of the BM-DC.

3. Materials and Methods

3.1 Chemicals

Media

- Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma-Aldrich) with 4500 mg/l glucose, 0.04 M $NaHCO_3$, 4 mM L-glutamine, 1 mM sodium pyruvate
- Roswell Park Memorial Institute 1640 Medium (RPMI) (Sigma-Aldrich) with 0.02 M $NaHCO_3$ and 2 mM L-glutamine

Medium Additives

- Fetal Calf Serum (FCS) (heat inactivated, Invitrogen)
- L-glutamine (Glu) (200 mM, Sigma-Aldrich)
- Penicillin (10 000 U) + Streptomycin (10 mg/ml) (Pen-Strep) (100x concentrated, Sigma-Aldrich)
- Non-essential amino-acids (NEA) (L-alanine, L-asparagine, L-aspartic acid, L-glycine, L-serine, L-proline and L-glutamic acid) (100x concentrated, Sigma-Aldrich)
- 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (1M, pH 7.0-7.6, Sigma-Aldrich)
- Glucose (Glc) (25 % solution in deionized water, prepared at Media and Glass Washing Facility at Institute of Molecular Genetics ASCR, v.v.i.)
- Sodium pyruvate (NaPyr) (100 mM, Sigma-Aldrich)
- 2-mercaptoethanol (2-ME) (50 mM, Sigma-Aldrich)

LMW Drugs and NO Donors

- Doxorubicin (Dox) (Meiji Seika Kaisha, LTD.)
- Adriblastina (Dox-HCl, 2 mg/ml, pharmaceutical form, Pfizer)
- Docetaxel (Dtx) (obtained from Institute of Macromolecular Chemistry ASCR, v.v.i.)

- Nitroglycerin (NG) (obtained from Institute of Macromolecular Chemistry AS-CR, v.v.i.)
- Sodium nitroprusside dihydrate (SNP)($\geq$ 98%, Fluka)

Other Chemicals

- Dimethyl sulfoxide (DMSO) (99.7 %, Sigma-Aldrich)
- Ethylenediaminetetraacetic acid (EDTA) (0.02 % solution in deionized water, prepared at Media and Glass Washing Facility at Institute of Molecular Genetics ASCR, v.v.i.)
- EDTA disodium salt (Na_2EDTA) (5 M, Invitrogen)
- Evans blue (EB) ($\geq$75%, Fluka)
- Formamide ($\geq$99%, Alfa Aesar)
- Trypsin (0.5 % solution in deionized water, prepared at Media and Glass Washing Facility at Institute of Molecular Genetics ASCR, v.v.i.)
- Thiazolyl Blue Tetrazolium Bromide (MTT) (98%, Sigma-Aldrich)

3.2 Solutions

Phosphate Buffer Saline (PBS)

9.0 g NaCl, 1.2 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g $NaH_2PO_4 \cdot 2H_2O$, 0.2 KCl, distilled water to volume 1000 ml and HCl for adjustment of pH to 7.4.

Flow Cytometry Buffer (FACS buffer)

PBS, 2 mM (Na_2EDTA), 2 % Fetal Calf Serum (FCS)

Trypsin-EDTA solution

0.5 % EDTA solution and 0.02 % trypsin solution combined in ratio 1:1

3.3 Cytokines

- Murine recombinant GM-CSF (rGM-CSF) (Peprotech)
- Murine IL-4 (Peprotech)

3.4 Antibodies for FACS Analysis

- anti-CD11c-PE.Cy7 (diluted 1:200, eBioscience)
- anti-MHC II-A700 (diluted 1:250,eBioscience)
- anti-CD11b-V500 (diluted 1:80, eBioscience)
- anti-CD40-PE (diluted 1:30, eBioscience)
- anti-CD80-FITC (diluted 1:500, eBioscience)
- anti-CD86-PerCP (diluted 1:300, Biolegend)
- anti-CD16/32 (diluted 1:50, eBioscience)

Titration of each conjugated mAb was performed prior to experiments on cells isolated from spleens of C57BL/6 mice.

3.5 Cell Lines

All the cell lines were purchased from ATCC. The cell lines and their species and tissue origin are described in Table 3.1. Culture conditions – media and medium additives for the cell lines are described in Table 3.2.

Cell line	**ATCC N°**	**Species**	**Strain**	**Tissue Origin**
EL4	TIB-39^{TM}	Murine	C57BL/6	T-cell lymphoma
EL4.IL-2	TIM-181^{TM}	Murine	C57BL/6	T-cell lymphoma
4T1	CRL-2539^{TM}	Murine	BALB/c	Breast carcinoma
EA.hy926	CRL-2922^{TM}	Human		Endothelial cell hybrid
RAW 264.7	TIB-71^{TM}	Murine	BALB/c	Abelson murine leukemia virus transformed macrophage

Table 3.1: **Cell lines and their origin.**

Cell line	Cultivation Medium	Medium Additives (per 100 ml of medium)
EL4	RPMI	10% FCS, 1 ml Pen-Strep, 2 ml Glu, 1 ml NaPyr, 1.8 ml Glc
EL4.IL-2	D-MEM	10% FCS, 1 ml Pen-Strep, 2 ml Glu
4T1	RPMI	10% FCS, 1 ml Pen-Strep, 1 ml Glu, 1 ml NaPyr, 1.8 ml Glc, 1 ml HEPES
EA.hy926	D-MEM	10% FCS, 1 ml Pen-Strep, 1 ml Glu
RAW 264.7	D-MEM	10% FCS, 1 ml Pen-Strep, 2 ml Glu

Table 3.2: **Culture media for maintaining of the cell lines.**

The cell line EL4 explored in *in vivo* tumor model does not incorporate thymidine during proliferation; its subline EL4.IL-2 was used instead for cytostatic/cytotoxic tests *in vitro*.

Adherent cell lines were detached from cultivation flasks using trypsin-EDTA solution. All the cell lines were centrifuged at 1200 rpm for 5 min at 4°C using Rotana 460R centrifuge (Hettich).

3.6 HPMA Copolymers

3.6.1 HPMA Copolymers with Organic Nitrates

The HPMA-based copolymers with organic nitrates (further assigned as polymer NO donors) were prepared and characterized in the Laboratory of Biomedicinal Polymers at the Institute of Macromolecular Chemistry ASCR, v.v.i. by Ing. Martin Studenovský, Ph.D.

Three different polymer NO donors were synthesized - conjugate 1, 2 and 3 (see Figure 3.1). These polymer NO donors vary in molecular weight and nitrate content (see Table 3.3). Conjugate 2 contains hydrolytically labile bond, whereas conjugates 1 and 3 contain hydrolytically stable bond by which organic nitrates are bound.

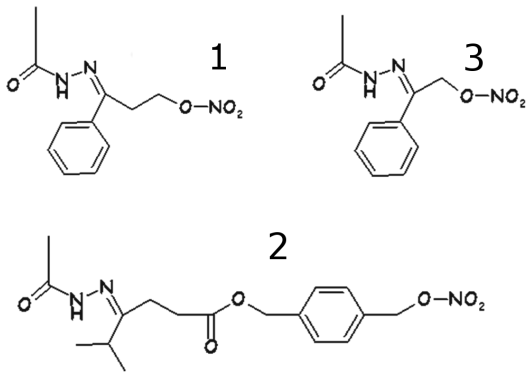

Figure 3.1: **Structure of organic nitrates attached to HPMA polymer carrier.**

Conjugate	NO donor bound by	M_w (g/mol)	M_w/M_n	Nitrate content (mmol/g)
Conjugate 1	hydrolytically stable bond	64,000	2.2	0.64
Conjugate 2	hydrolytically labile bond	55,000	2.1	0.22
Conjugate 3	hydrolytically stable bond	41,000	1.78	0.37

Table 3.3: **HPMA-based conjugates with NO donors (organic nitrates).**

3.6.2 HPMA Copolymer with Dox

For *in vivo* tumor treatments, we used star-like HPMA-based copolymer with Dox. This copolymer consists of semitelechelic HPMA-based polymers with Dox attached by hydrazone bond, that are conjugated with 2^{nd} generation dendrimer core. This polymer was prepared as previously described (Etrych *et al.*, 2011). Further, this copolymer will be assigned as star-like pHPMA-Dox or star-Dox in Figures.

Drug	M_w (g/mol)	M_w/M_n	Dox (wt %)	Free Dox (wt %)	R_h (nm)
Star-like pHPMA-Dox	250,000	1.50	10.7	< 0.2 %	13±2

Table 3.4: **Characteristics of star-like HPMA-based copolymer with Dox.**

3.7 Proliferation Assay

Testing of the cytostatic or cytotoxic effect of the polymer conjugates and LMW drugs was performed by H^3-thymidine incorporation assay or MTT assay. The cells were seeded to 96-well flat bottom tissue plates (Thermo Fisher Scientific). 5×10^5 EL4.IL-2, EL4 and EA.hy926 and 2.5×10^5 4T1 cells per well were used.

Various concentrations of drugs/NO donors were added to the cells at a total volume of 250 µl. The chemotherapeutic drugs were diluted in sterile PBS to concentration 400 mg/ml, while LMW and polymer NO donors were diluted in DMSO to 5 mM nitrate concentration. Further dilution of drugs and NO donors was performed using cultivation medium. The measurement for each sample concentration was performed in quadruplicates. For all the *in vitro* experiments, NG was used as positive control for NO release.

The cells were incubated for 72 hours in 5% CO_2 atmosphere at 37°C in CO_2 incubator (Sanyo).

Because RPMI medium contains high level of gluthatione, which we suppose can interfere with biotransformation of organic nitrates to NO. Therefore, for incubation with NO donors, the culture conditions were adopted for using D-MEM. For this reason, normal murine splenocytes could not be explored for tests of NO donor toxicity, as they do not proliferate in D-MEM even in the presence of a mitogen such as concanavalin A (2.5 and 5 µg/ml).

3.7.1 H^3-thymidine Incorporation Assay

After the cultivation, H^3-thymidine was added to each well in 50 µl of culture medium with final concentration 37 kBq per well and was incubated for 7 more hours. Samples were then frozen at -20°C overnight.

When the samples reached the room temperature, the cells were harvested using Mach III harvestor (Tomtec) on glass fiber filters Filtermat (Perkin Elmer). The radioactivity of the samples was measured in scintillation counter 1450 MicroBeta Trilux (Wallac), using solid scintillator Meltilex (Perkin Elmer). Cells cultivated in fresh medium were used as controls. The cytostatic effect of substances tested was determined as value of inhibition of the cell growth by 50% (IC_{50} value).

3.7.2 MTT Assay

After the cultivation, cells were incubated in 80 µl media together with 20 µl MTT solution (5 mg/ml in PBS) for approximatelly 2 hours. DMSO (200 µl) was added after the cultivation and absorbance was measured using microplate reader Infinite M200 Pro (Tecan Group Ltd.) at 540 nm with reference wavelength 690 nm. The cytotoxic effect of substances tested was determined as value of inhibition of the cell metabolic activity by 50% (IC_{50} value).

3.8 Experimental Murine Tumor Models

Two inbred mice strains, C57BL/6 (*H-2*^{b}) and BALB/c (*H-2*^{d}) were obtained from Institute of Physiology ASCR, v.v.i. (Prague). All mice used were females 8-16 weeks old; they were housed in conventional conditions with access to food and water *ad libitum*.

Two tumor models, EL4 T-cell lymphoma and 4T1 breast carcinoma, were used for experiments. Only the cells that had viability higher than 90 % were used. The cells were cultivated to high, but not maximum confluency; 1x10^5 EL4 or 5x10^5 4T1 cells were injected s.c. on mouse back.

The tumor size was measured by calipers and the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Tumor volume was then calculated as $V = \frac{\mathrm{length} \times \mathrm{width}^2}{2}$. The tumors were allowed to grow to size of approximately 200 mm^3 (day 8 after injection) before any experiment was started.

3.9 Testing of Systemic Toxicity

Tumor-free C57BL/6 mice were used for assessing systemic toxicity of polymer NO donors. Various doses of polymer NO donors were tested and body weight loss was considered as the main indicator of toxicity. Incidental discomfort was monitored by fur erection, hunched posture, food and water intake and overall muscle tonus. Polymer NO donors were diluted in sterile PBS or FCS-free medium to desired concentration. Maximum tolerated dose was set as the concentration that causes 15 % weight loss.

3.10 Accumulation of Macromolecules and Drugs in Tumor

3.10.1 Evans Blue Assay

To detect whether polymer NO donors affect accumulation of macromolecules in the tumor, Evans blue (EB) — LMW stain with high affinity for serum albumin was administered to the tumor-bearing mice (day 8 after tumor injection) i.v. at concentration of 10 mg per kg of mouse weight. EB and polymer NO donors were diluted in sterile PBS to desired concentration. The conjugate 1 (eq. 10-100 µmol nitrate/kg) and conjugate 2 (eq. 100 µmol nitrate/kg) were administered i.v. within ten minutes after EB injection.

The mice were sacrificed and exsanquinated by interruption of carotid artery 6, 12, 24 and 48 hours after injection of EB. Subsequently, the tumors were dissected, weighted and cut into small pieces that were placed in formamide (1 ml of formamide per 100 mg of tissue), in which extraction of EB was performed for 3 days at room temperature in the darkness. Afterwards, 200 µl of the supernatant was transferred to the 96-well flat-bottomed black plates (Thermo Fisher Scientific) and fluorescence (ex. 620 nm, em. 680 nm) was measured using microplate reader Infinity M200 PRO (Tecan Group Ltd.).

3.10.2 Dox Content in Tumor

The EL4 T-cell lymphoma-bearing C57BL/6 mice were treated with star-like pHPMA-Dox (eq. 7.5 mg Dox/kg, i.v.) at day 8 after tumor cell injection. Conjugate 1 was administered i.v. to these mice in four doses (20 µmol nitrate/kg each) at time intervals -12, 0, 24 and 48 hours respective to injection of star-like pHPMA-Dox. Star-like pHPMA-Dox and polymer NO donors were diluted in sterile PBS to desired concentration. The mice were sacrificed 12, 24 and 72 hours after the star-like pHPMA-Dox injection (see Figure 3.2).

The tumor, muscle and liver samples were then dissected from each mouse, weighted and frozen to -20°C. The samples were transferred to IMC ASCR v.v.i., where content of Dox in the samples was measured using high-performance liquid chromatography (HPLC) as previously described (Etrych *et al.*, 2008; Chytil *et al.*, 2015). The results are expressed in µg Dox per g of tumor tissue and they represent sum of Dox bound to polymer and free Dox.

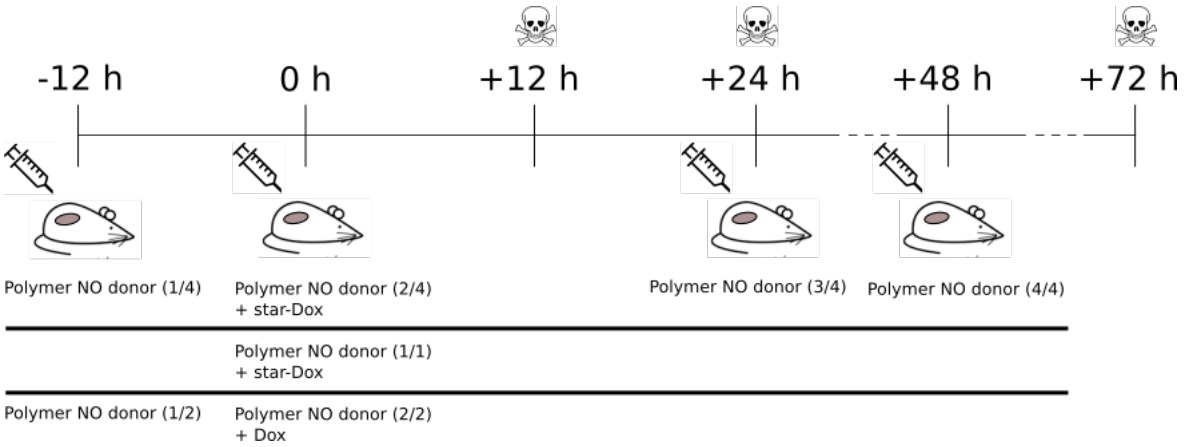

Figure 3.2: **Treatment and sacrification scheme in C57BL/6 mice.**

3.11 Effect of Polymer NO Donors on Treatment of Murine Experimental Tumors

The C57BL/6 mice bearing EL4 tumors were treated by star-like pHPMA-Dox (eq. 7.5 mg Dox/kg) or LMW Dox (7.5 mg/kg). The treatment was administered at day 8 after tumor cell injection. The sub-optimal dose of the star-like pHPMA-Dox was administered to put the treatment outcome at 25-50% tumor-free mice at day 60, so that we could observe potential improvement or even worsening of the treatment. The mice further received either conjugate 1 or 2 in four doses (eq. 20 µmol nitrate/kg) at times -12, 0, 24 and 48 hours, or in single dose (eq. 80 µmol nitrate/kg) at time 0 hours respective to administration of the star-like pHPMA-Dox (see Figure 3.2).

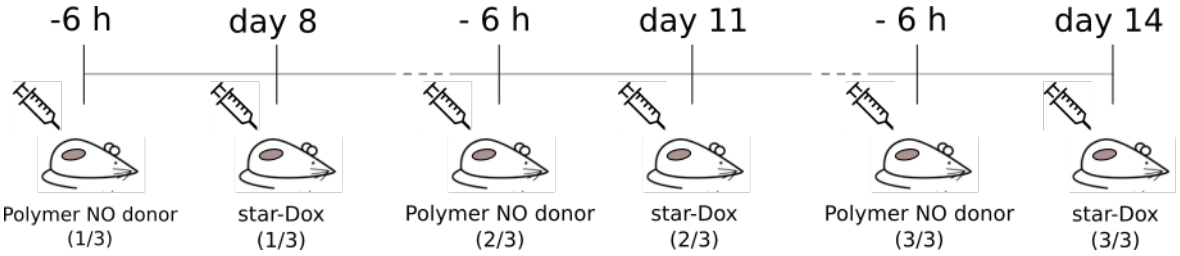

Figure 3.3: **Treatment scheme in BALB/c mice.**

The BALB/c mice bearing 4T1 tumors were treated by three doses of star-like pHPMA-Dox (eq. 8 mg Dox/kg each), that has been more effective than treatment by one dose in previous experiments. The treatment was administered at day 8, 11 and 14 after tumor cell injection. Additional treatment with Conjugate 1 was also delivered in three doses (eq. 20 µmol nitrate/kg), each 6 hours before administration of each dose of the star-like pHPMA-Dox (see Figure 3.3).

Star-like pHPMA-Dox and polymer NO donors were diluted in sterile PBS to desired concentration. Tumor growth was measured as described in section 3.2.3 and together with survival rate, it was observed in experiments with both murine models. For each group, 8 mice were used. The data were analyzed using Graphpad Software and survival curves were compared using longrank test.

3.12 Generation and Stimulation of Murine BM-DC

Murine bone marrow derived dendritic cells (BM-DC) were prepared by isolation of bone-marrow cells from femurs of C57BL/6 mice and their differentiation towards DC phenotype was conditioned by GM-CSF and IL-4 cytokines.

The mice were sacrificed by cervical dislocation and the femurs were extracted. The bones were superficially cleaned using gauze soaked in 70% EtOH. Subsequently, the femurs were disinfected from any possible contaminations in 70% EtOH for approximately 1 minute. Afterwards, they were placed into sterile PBS. The epiphyses were cut using sterile scissors and the content of the bone was flushed using 2 ml syringe with 0.25 mm needle filled with PBS with 1% of FCS. The cells were subsequently mechanically separated using pipette and passed through 70 µm strainer (Thermo Fisher Scientific).

The cells were plated at 6-well culture plates (5×10^6 cells/well) to 5 ml of RPMI medium . The medium was supplemented with 0.1 ml 2-ME, 1 ml Glu, 1 ml Pen-Strep and 1 ml NEA per 100 ml. Murine rGM-CSF (20 ng/ml) and murine IL-4 (10 ng/ml) were added to the medium right before the experiment. After 48 hours, additional 5 ml of the culture media were added to each well. After next 48 hours, half of the medium was collected from each well, centrifuged, supernatant was discarded and 5 ml of fresh medium was added to the cell suspension, which was then returned to the original cultivation well. After next 72 hours, the non-adherent and semi-adherent cells were collected and used for further stimulation/analysis. Total period of BM-DC cultivation was 7 days.

The BM-DC were afterwards pipetted into 96-well U-bottomed plates (Thermo Scientific) ($1x10^6$ cells/well), incubated with several concentrations of conjugate 1 (eq. 0.02-20 µM nitrate/kg), and 1 µg/ml LPS was used as positive control of stimulation. Moreover, stimulation of BM-DC by the sames conjugate 1 concentrations in combination with LPS was also tested. After 20 hours of incubation, the cells were flushed with fresh medium and subjected to staining and flow cytometry analysis.

3.13 FACS Analysis

The BM-DC were transferred to FACS buffer, the Fc receptors on the cells were blocked using anti-CD16/32 mAb diluted 50x according to manufacturer recommendation for 10-20 minutes. The staining solutions were prepared in FACS buffer using fluorophore-conjugated mAbs listed in section 3.1.4. Next, the cells were washed with FACS buffer and prepared staining solutions of conjugated mAbs were added to corresponding wells. The cells were incubated with the staining mAbs for 30 min on ice in the dark. Afterwards, the cells were washed twice using FACS buffer and kept on ice. Right before analysis on LSR II flow cytometer (BD Biosciences), Hoechst 33258 viability stain (0.1 g/ml) was added to each sample. At least 50,000 cells were analyzed for each sample and FlowJo software (Tree Star, Inc.) was used for analysis. Live (Hoechst negative), $CD11c^{+}$ MHC II^{+} $CD11b^{int}$ $CD115^{-}$ cells were considered BM-DC, as described recently (Helft *et al.*, 2015).

3.14 Data Analysis

Data from most experiments were analyzed using Graphpad Software and mean ± S.D. values of measurement were displayed. For statistical analysis, Student's t-test for independent measurements was used, when not stated differently. A value of $p < 0.05$ was considered significant.

4. Results

4.1 Cytostatic Activity of Polymer NO Donors

Firstly, cytostatic activity of sole polymer NO donors was tested on murine T-cell lymphoma line EL4.IL-2, murine breast cancer cell line 4T1, and human endothelial cell line EA.hy926. The EL4.IL-2 cell line was tested as thymidine-incorporating subline of EL4 cells that were used in the experimental *in vivo* model together with 4T1. The EA.hy926 cell line was used as a representation of endothelial cells, an important constituent of tumor neovasculature. Although this line is immortalized, it can be considered an adequate cell type.

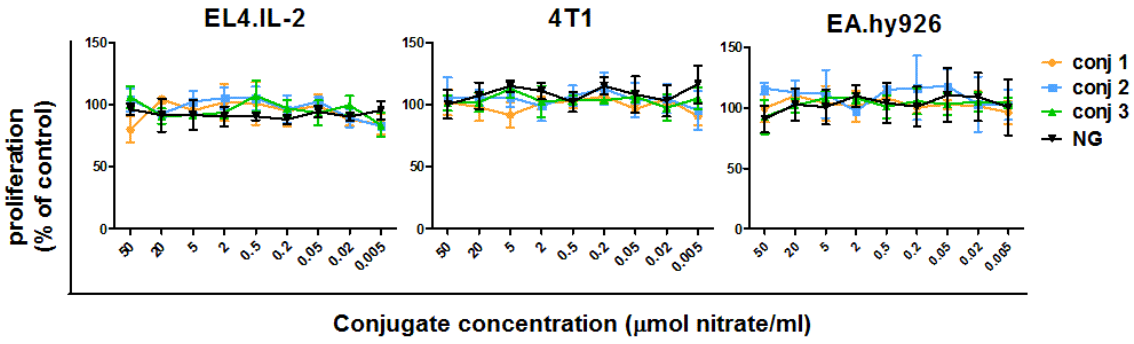

Figure 4.1: **Cytostatic activity of NG and three polymer NO donors on permanent cell lines.** The cell lines EL4.IL-2, 4T1 and EA.hy926 were cultivated with polymer NO donors in concentrations equivalent to 0.005–50 µmol nitrate/ml in D-MEM. NG was used as positive control. H^3-thymidine incorporation assay was performed after 72 hours of incubation. Results are expressed as a percentage of control (untreated cells).

Neither polymer NO donors, nor NG, used as LMW NO donor for control, had significant cytostatic activity towards the cell lines EL4.IL-2, 4T1 and EA.hy926 (see Figure 4.1). We have also tested the cytotoxic activity of polymer NO donors using MTT assay and we did not observe any cytotoxic effect of NG or polymer NO donors on the three selected cell lines (data not shown).

Drug	EL4.IL-2 IC_{50} ($ng\,ml^{-1}$)	4T1 IC_{50} ($ng\,ml^{-1}$)	EA.hy926 IC_{50} ($ng\,ml^{-1}$)
Dox	13.10±4.96	1.68±5.12	22.83±7.47
Dtx	3.57±1.03	1.64±0.58	0.51±0.25

Table 4.1: **IC_{50} values of Dox, Dtx, star-like pHPMA-DOX.** IC_{50} values represent concentration of drug (Dox, Dtx and star-like pHPMA-Dox) which inhibits proliferation of cells by 50%. Three cell lines were used for detection of cytostatic activity of the drugs - murine T lymphoma (EL4.IL-2), breast carcinoma (4T1) and human endothelial cells (EA.hy926).

4.2 Effect of Polymer NO Donors on Cytostatic Activity of Dox and Dtx *in vitro*

NO has been previously argued to influence the cytostatic effect of anthracyclines in human breast carcinoma and colon carcinoma cells (Evig *et al.*, 2004; Muir *et al.*, 2006; De Boo *et al.*, 2009). To examine this hypothesis in the selected murine cell lines, we performed H^3-thymidine proliferation assays, where we explored the effect of NO donors on cytostatic activity of Dox and Dtx.

In order to set up conditions for the experiments, we first measured the cytostatic activity of the mentioned drugs, determining the IC_{50} values of each chemotherapy used - Dox and Dtx (see Table 4.1).

Exploring the effect of chemosensitization of the cells towards treatment by Dox or Dtx, we incubated the EL4.IL-2, 4T1 and EA.hy926 cells with a combination of the drugs and the polymer NO donors or NG as a positive control. We used drug concentrations which inhibited cell proliferation approximately by 10, 25, 50 and 75%. The concentrations of polymer NO donors were used in a wide range from those eq. to 0.005 µmol nitrate/ml to 20 µmol nitrate/ml.

Despite that we used the whole range of concentrations of polymer NO donors and NG, the polymer NO donors and either NG did not have any effect on cytostatic activity of the drugs towards the EL4.IL-2, 4T1 and EA.hy926 cell lines (see Figures 4.2, 4.3, 4.4, 4.5).

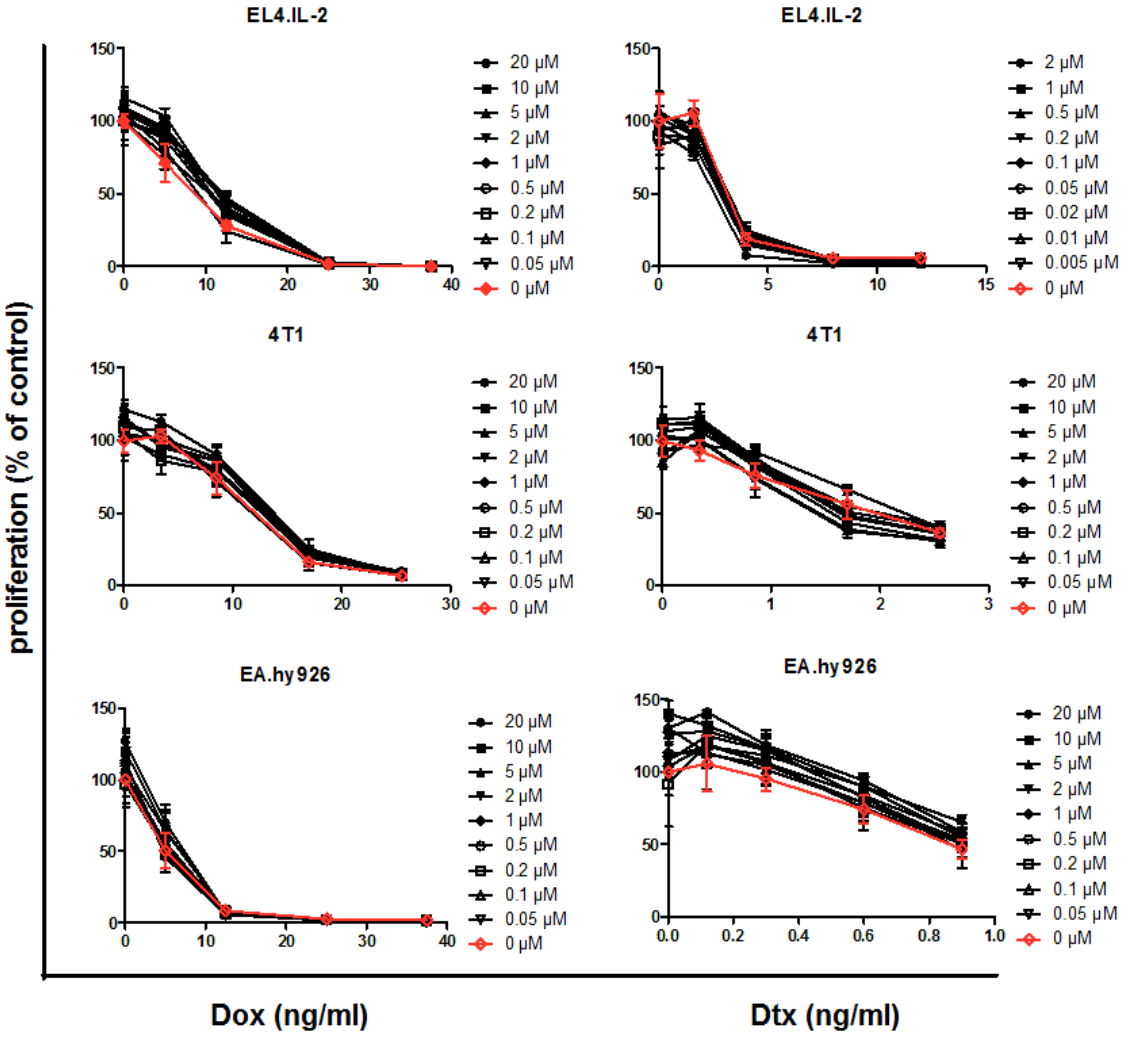

Figure 4.2: **Chemosensitisation by NG towards Dox and Dtx detected in three cell lines.** EL4.IL-2, 4T1 and EA.hy926 cells were cultivated with Dox or Dtx and NG and H^3-thymidine incorporation assay was performed. Incubation lasted a total of 72 hours. Dox and Dtx concentrations were set according to IC_{50} value in each cell line. NG concentrations ranging from 0.005 to 20 µM were tested.

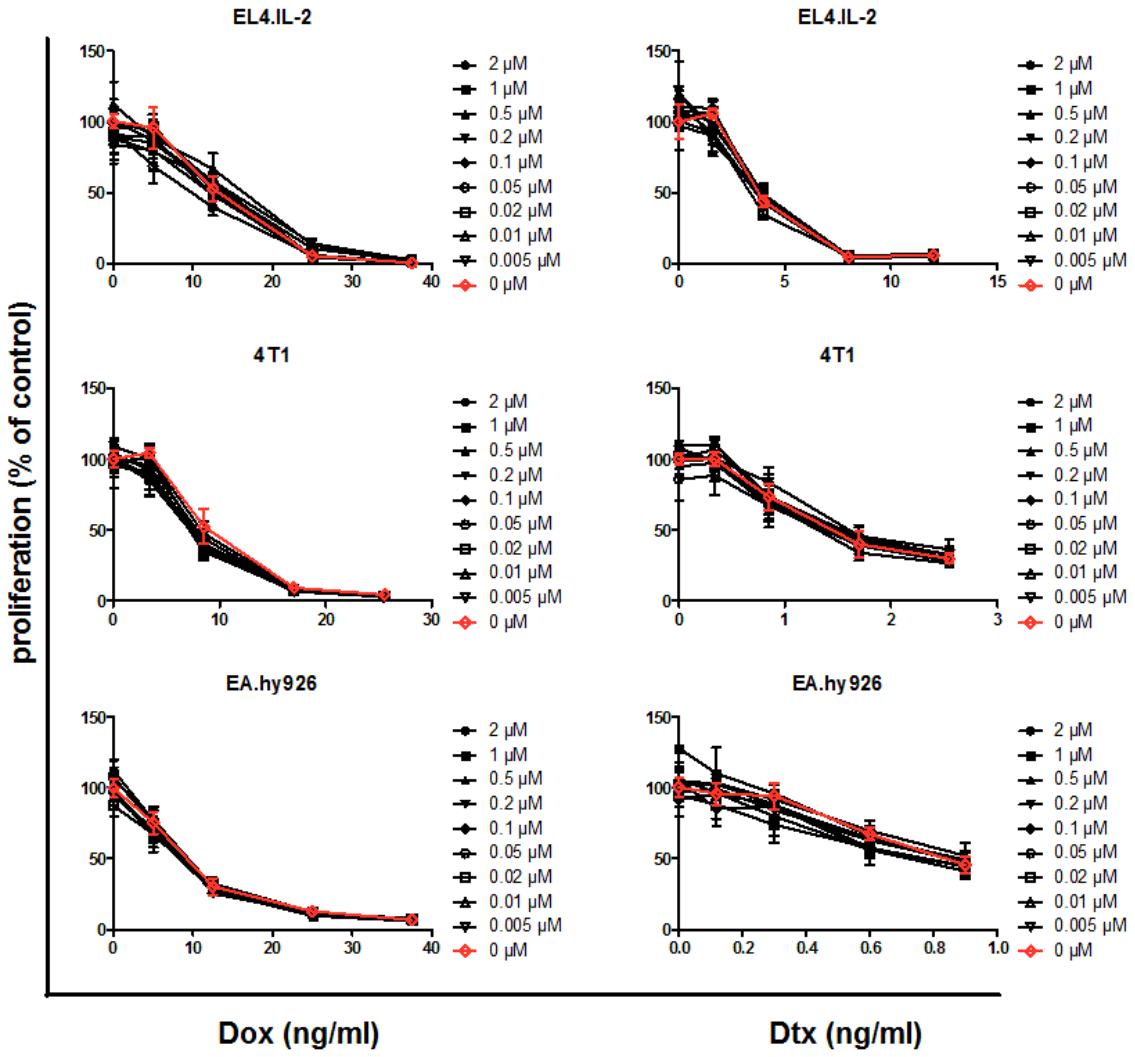

Figure 4.3: **Chemosensitisation by conjugate 1 towards Dox and Dtx detected in three cell lines.**EL4.IL-2, 4T1 and EA.hy926 cells were cultivated with Dox or Dtx and conjugate 1 and H^3-thymidine incorporation assay was performed. Incubation period lasted a total of 72 hours. Dox and Dtx concentrations were set according to IC_{50} value in each cell line. Conjugate 1 concentrations ranging from 0.005 to 20 µM nitrate were tested.

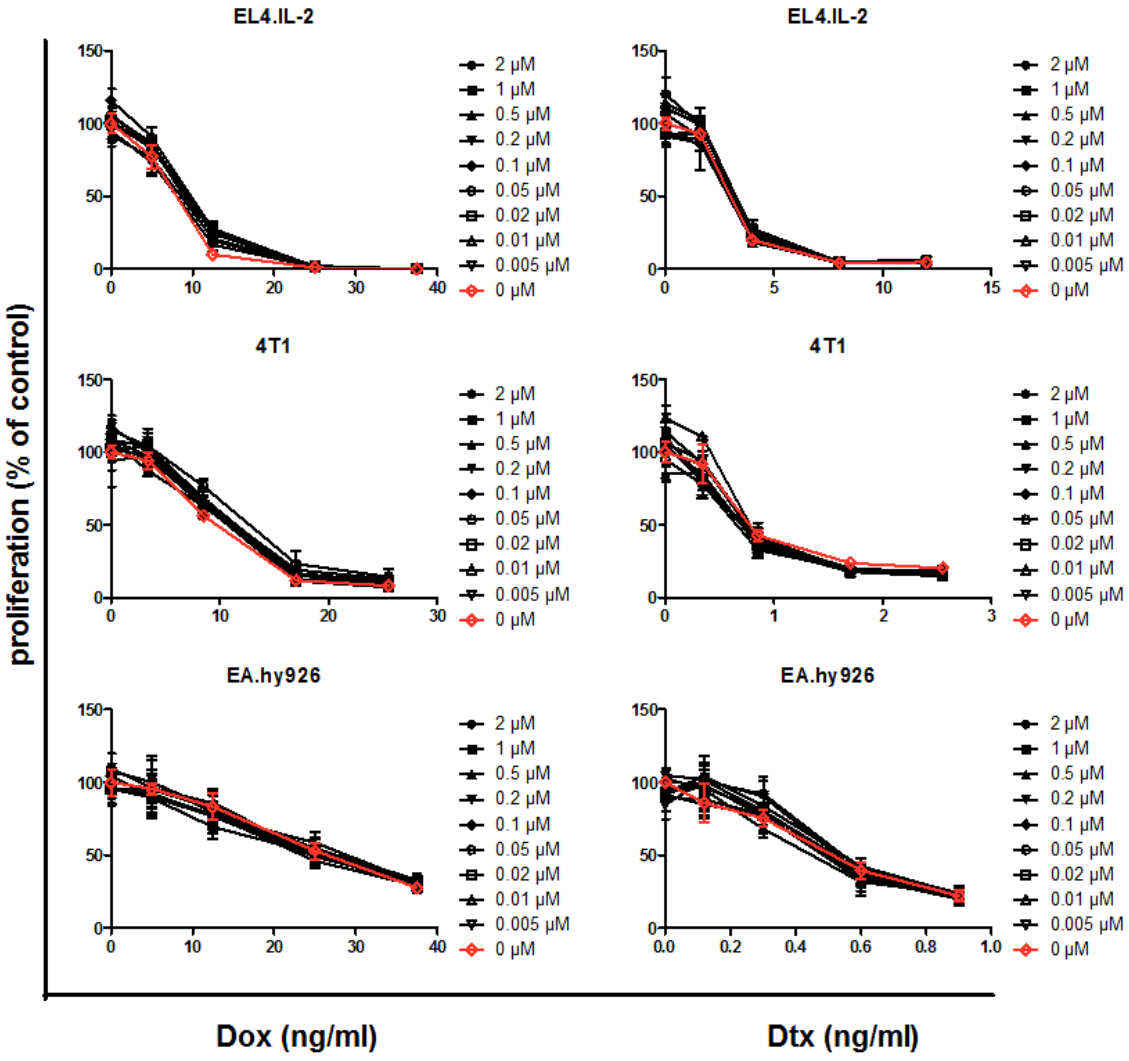

Figure 4.4: **Chemosensitisation by conjugate 2 towards Dox and Dtx detected in three cell lines.** EL4.IL-2, 4T1 and EA.hy926 cells were cultivated with Dox or Dtx and conjugate 2 and H^3-thymidine incorporation assay was performed. Incubation period lasted a total of 72 hours. Dox and Dtx concentrations were set according to IC_{50} value in each cell line. Conjugate 2 concentrations ranging from 0.005 to 20 µM nitrate were tested.

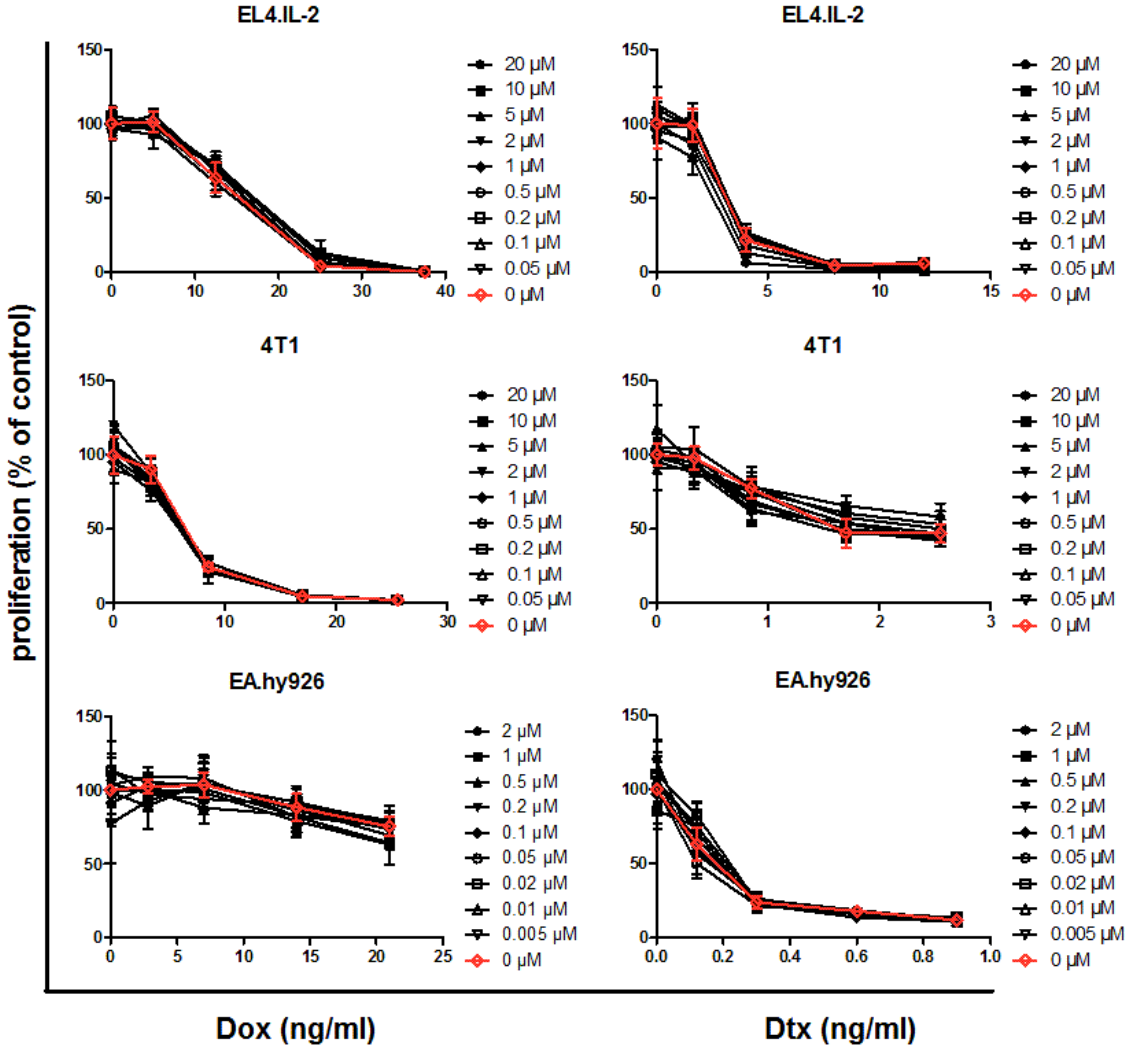

Figure 4.5: **Chemosensitisation by conjugate 3 towards Dox and Dtx detected in three cell lines.** EL4.IL-2, 4T1 and EA.hy926 cells were cultivated with Dox or Dtx and conjugate 3 and H^3-thymidine incorporation assay was performed. Incubation period lasted a total of 72 hours. Dox and Dtx concentrations were set according to IC_{50} value in each cell line. Conjugate 3 concentrations ranging from 0.005 to 20 µM nitrate were tested.

4.3 Systemic Toxicity of Polymer NO Donors

Before any *in vivo* experiments, the release of NO from the polymer NO donors was analyzed in our laboratory. The release of NO from two of the polymer NO donors – conjugate 1 and 2 – was verified. This analysis was performed by a method adopted from the Nitric Oxide Fluorometric Cell-Based Assay Kit (Cayman Chemicals) using direct intracellular NO detection by fluorometric probe FA-OMe. All the measurements were carried out by Mgr. Ladislav Sivák and have not yet been published. These data showed, that the two conjugates release NO peaking in concentration eq. to 0.02 – 0.05 µM nitrate. A similar trend was observed in NG, when the highest NO release was observed at the concentration of 0.02 µM. As NO release was detected only in conjugates 1 and 2, we did not use conjugate 3 in further experiments.

LMW donors of NO, such as sodium nitroprusside (SNP) are lethal in relatively small doses of approximately 20 µm (Hospira Inc., 2011). We examined the toxicity of the two polymer NO donors (conjugate 1 and 2), tested several concentrations of the polymer NO donors and also checked various treatment regimes. Even a dose equal to 150 µmol nitrate/kg did not cause body weight loss (see Figure 4.6), which was set as the primary parameter of systemic toxicity. The results showed that the polymer NO donors at the dose of 150 µmol nitrate/kg do not even approach the threshold of the maximum tolerated dose, which was set as 15% loss in body weight. Moreover, none of the mice exhibited indications of discomfort immediately after administration or during the whole monitoring period.

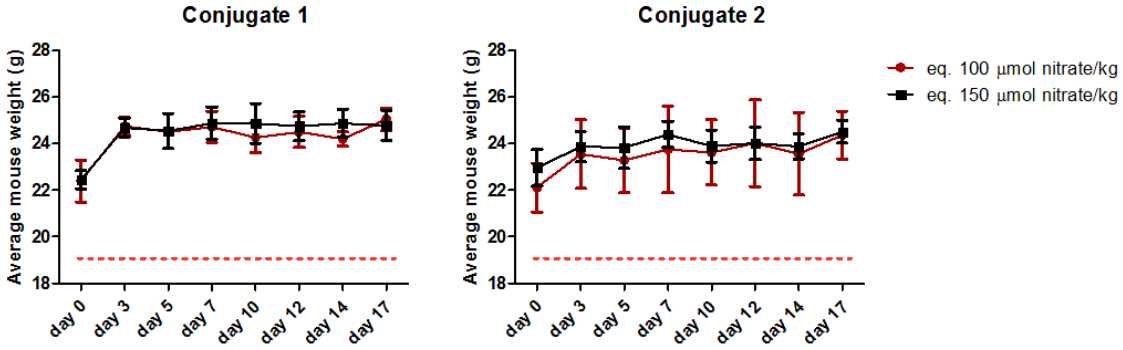

Figure 4.6: **Effect of polymer NO donors on average body weight of C57BL/6 mice.** After i.v. administration of conjugates 1 and 2, average body weight was observed for 17 days. Conjugates were administered in doses of 100 and 150 µmol nitrate/kg. The red line represents a 15% decrease in weight, which is considered a limit value of the maximum tolerated dose. In each group, 3 mice were tested.

4.4 Accumulation of Macromolecules in Tumor — Evans Blue Assay

The next step was to demonstrate potential vasodilation by polymer NO donors, resulting in an increase of accumulation of the macromolecules. Evans blue (EB) — a LMW stain with a high affinity for serum albumin was used as the detection probe for the accumulation of macromolecules in the tumor. As murine serum albumin is approximately 67 kDa in size, exceeding the 40 kDa threshold, it is subjected to the EPR effect. Administered to the circulation, EB binds very quickly and quantitatively to serum albumin and allows for the detection of the EPR effect in solid tumors (Matsumura & Maeda, 1986). Using this method, we tested whether conjugate 1 can influence the accumulation of macromolecules in tumor. A relatively small dose of conjugate 1 (10 µmol nitrate/kg) was tested in the first experiments.

As the results showed, in BALB/c mice bearing 4T1 tumor there was approximately the same level of EB in the tumors 6 and 12 hours after the injection of EB or conjugate 1 together with EB. However, a statistically significant enhancement of EB accumulation in the group that was receiving conjugate 1 with EB occurred after 24 hours (see Figure 4.7). The mice were bearing two tumors induced by two s.c. applications of tumor cells in order to obtain more samples for analysis. The tumors did not show any inter-dependency within one mouse, considering both tumor weight and EB fluorescence intensity. Therefore, all the tumors were evaluated as independent samples.

As the trend of increased accumulation was observed at the time of 24 hours, we repeated the experiment, shifting the time frame to 48 hours. When EB was co-administered with conjugate 1, a moderate increase of EB content was observed at 12 hours and 48 hours, but no significant difference was detected between mice that received EB and those which received both EB and conjugate 1 after 24 hours (see Figure 4.8). Thus, we did not fully confirm the results from the previous experiment.

We then also tested EB-albumin complex accumulation in the murine model of EL4 lymphoma. Here, we used 10 times higher dose of conjugate 1 and conjugate 2 (100 µmol nitrate/kg). The results showed no significant increase in the accumulation of EB-albumin complex in the tumor after 6, 12, 24, and 48 hours. By contrast, a significant decrease in the accumulation of this complex was detected 24 hours after the administration of conjugate 1 and also 48 hours after the administration of conjugate 2 (see Figure 4.9).

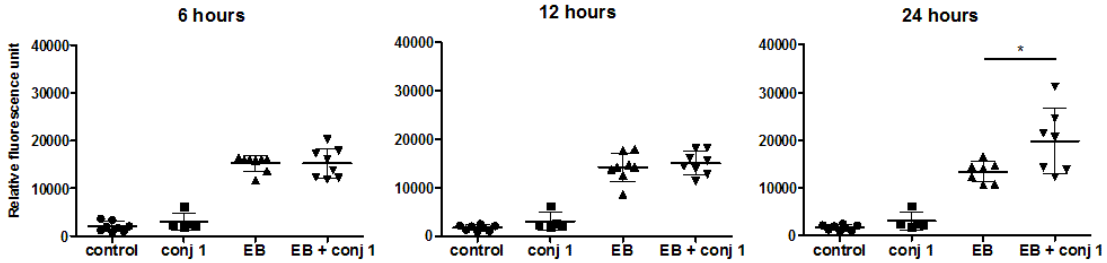

Figure 4.7: **Accumulation of EB bound to serum albumin in murine breast carcinoma (4T1) tumor model.** Fluorescence of EB in 4T1 tumors from BALB/c mice was measured 6, 12 and 24 hours after i.v. injection of conjugate 1 (10 µmol nitrate/kg of mouse weight) and EB (10 mg/kg). For each group 4, mice with two tumors were used. Each tumor was measured and evaluated as an independent sample. Mean ± SD values are displayed for each group. Samples were evaluated by Student's t-test and significant difference was set at $p<0.05$ (*).

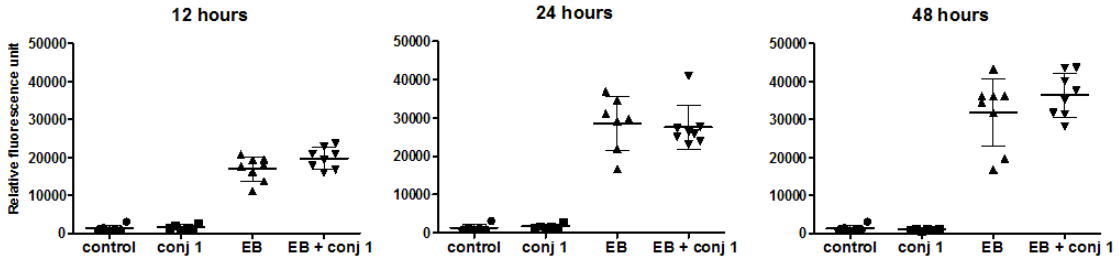

Figure 4.8: **Accumulation of EB bound to serum albumin in murine breast carcinoma (4T1) tumor model.** Fluorescence of EB in 4T1 tumors from in BALB/c mice was detected 12, 24 and 48 hours after injection of conjugate 1 (10 µmol nitrate/kg of mouse weight) and EB (10 mg/kg). For each group, 4 mice with two tumors were used. Each tumor was measured and evaluated as an independent sample. Mean ± SD values are displayed for each group.

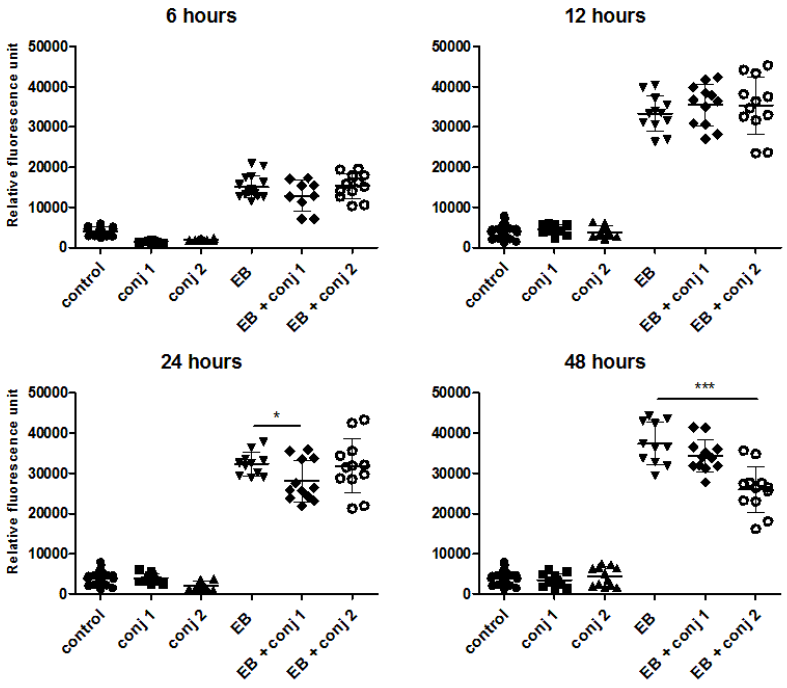

Figure 4.9: **Accumulation of EB bound to serum albumin in murine T lymphoma (EL4) tumor model.** Fluorescence of EB in EL4 tumors from C57BL/6 mice was detected 6, 12, 24 and 48 hours after injection of conjugate 1 (100 µmol nitrate/kg of body weight) and EB (10 mg/kg). For each group 3 mice with two tumors were used. Each tumor was measured and evaluated as an independent sample. Mean ± SD values are displayed for each group. Data were evaluated by Student's t-test and significant difference was set at $p<0.05$ (*), $p<0.001$ (***).

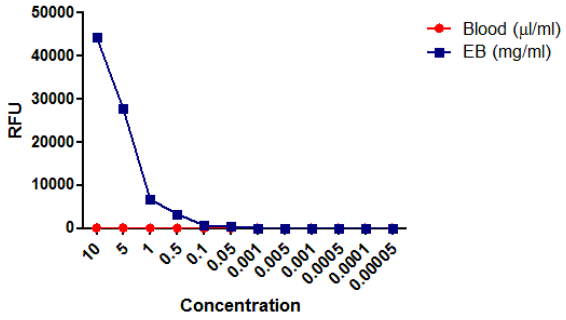

Figure 4.10: **Blood and EB fluorescence.** Fluorescence of various concentrations of heparinized blood from a healthy C57BL/6 mouse and various concentrations of EB, both diluted in formamide was measured at ex. 620 nm, em. 680 nm in 96-well black plates. EB was used as positive control.

As the polymer NO donors showed inconsistent results in accumulation of EB bound to serum albumin, we tried to figure out whether there is a substance that would interfere with fluorescence of EB that was measured. Supposedly, the polymer NO donors cause increased vasodilation and thus increased blood supply in tumor. Hence, we examined whether an increased content of blood in the tumor can interfere with the EB fluorescence of samples, creating false positive results. We diluted a sample of blood from a healthy C57BL/6 mouse in formamide and measured its fluorescence (see Figure 4.10). The results clearly showed that the blood fluorescence level was low, thus it most probably does not interfere with EB fluorescence at all.

4.5 Accumulation of Dox in Tumor

Considering that the EB assay did not unambiguously identify an increase in the EPR effect, probably not being robust enough, we applied a second option for the detection of potential intensification of the EPR effect by polymer NO donors. The C57BL/6 mice bearing EL4 tumors were treated with one dose of star-like pHPMA-Dox conjugate (eq. 7.5 mg/kg). Four doses (20 µmol nitrate/kg) of conjugate 1 were applied at time -12, 0, 24 and 48 hours respective to administration of star-like pHPMA-Dox conjugate. The mice were sacrificed 12, 24 and 72 hours after the star-like pHPMA injection (see Figure 3.2).

The accumulation of Dox in tumor and muscle samples was then measured in the Laboratory of Biomedicinal Polymers at the Institute of Macromolecular Chemistry ASCR v.v.i., using HPLC as described before (Etrych *et al.*, 2008). The results showed overall higher accumulation of Dox in tumors caused by conjugate 1 co-administration.

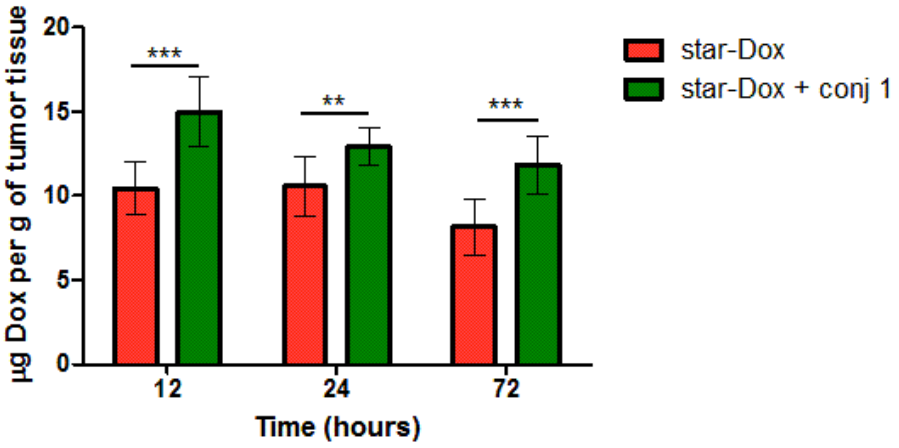

Figure 4.11: **Accumulation of Dox in EL4 tumors in C57BL/6 mice.** Four doses of conjugate 1 (20 µmol nitrate/kg each) were administered at times -12, 0, 24 and 48 hours respective to administration of star-like pHPMA-Dox conjugate (dose eq. 7.5 mg Dox/kg). Dox content in EL4 tumors was detected by HPLC at the Institute of Macromolecular Chemistry. Four mice per group were used. The results are represented as mean ± SD values of Dox content in the tumor.

The increase of almost 5 µg Dox per gram of tumor tissue caused by conjugate 1 at the time interval of 12 hours was statistically significant when compared to treatment without conjugate 1 (see Figure 4.11). The statistically significant increase in Dox accumulation in tumor caused by conjugate 1 can be also seen at 24 and 72 hours after treatment. These results proved that conjugate 1 indeed increases drug accumulation in tumors.

Furthermore, tumor-to-muscle and tumor-to-liver ratios of Dox content were calculated. Co-administration of conjugate 1 with star-like pHPMA-Dox significantly improved the preferential accumulation in tumor compared to muscle, and slightly improved the ratio of accumulation in tumor compared to liver. Content of Dox in liver was relatively high when compared to muscle, but, at 24 and 72 hours after treatment, lower than that in the tumor (see Figure 4.12).

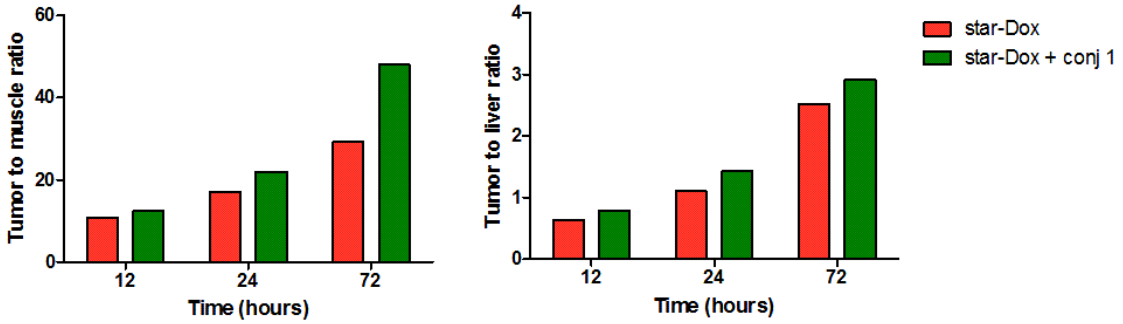

Figure 4.12: **Tumor-to-muscle (left) and tumor-to-liver (right) ratios of Dox content in C57BL/6 mice bearing EL4 tumor.** Four doses of conjugate 1 (20 µmol nitrate/kg each) were administered at times -12, 0, 24 and 48 hours respective to administration of star-like pHPMA-Dox conjugate (dose eq. 7.5 mg Dox/kg). Tumor-to-muscle/liver ratios were calculated from the Dox content (µg per gram of the tissue). Content of Dox in EL4 tumor, muscle and liver samples from C57BL/6 mice was detected by HPLC at the Institute of Macromolecular Chemistry.

4.6 Effect of Polymer NO Donors on Polymer-Based Chemotherapy of Murine EL4 T-Cell Lymphoma

In order to identify whether polymer NO donors influence the outcome of HPMA-based chemotherapy treatment, star-like pHPMA-Dox with molecular weight 250 kDa and hydrodynamic radius of 13 nm was used. Firstly, we used a tumor model in which we were able to treat the tumor completely, and set the dosage of treatment so that there would be 25-50% of tumor-free mice after 60 days. This time frame represents approximately twice the survival time period of untreated mice, and we consider it an arbitrary threshold for a complete cure. The dose administered to mice for the selected treatment outcome was eq. 7.5 mg Dox/kg. In one group of mice, four doses of conjugate 1 (20 µmol nitrate/kg each) were injected at -12, 0, 24 and 48 hours respective to star-like pHPMA-Dox treatment. In second group, single dose of conjugate 1 (80 µmol nitrate/kg) was administered simultaneously with the treatment (see Figure 3.2).

Results showed that the growth of the tumors was not influenced by conjugate 1 alone when compared to control. Also, co-administration of four doses or a single dose of conjugate 1 together with star-like pHPMA-Dox did not cause a difference in the tumor growth after treatment with star-like pHPMA-Dox alone. The co-administration of conjugate 1 with LMW Dox did not change tumor growth that was observed with

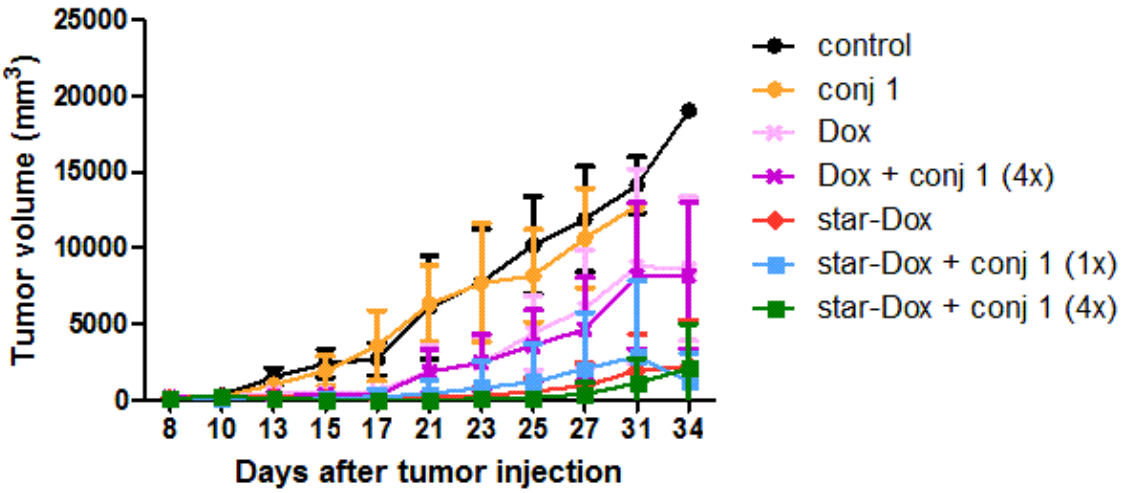

Figure 4.13: **Tumor growth of EL4 T lymphoma bearing C57BL/6 mice treated by star-like pHPMA-Dox and conjugate 1.** Four doses of conjugate 1 (20 µmol nitrate/kg each) at times -12, 0, 24 and 48 hours or 1 dose of conjugate 1(80 µmol nitrate/kg) at time 0 hours respective to administration of star-like pHPMA-Dox conjugate (labeled as star-Dox; dose eq.7.5 mg Dox/kg) were administered i.v.. Treatment by Dox (7.5 mg/kg) and its combination with same 4 doses of conjugate 1 as described above was also tested. Tumor volume mean ± SD values are displayed.

Dox treatment (see Figure 4.13).

Although tumor growth was not significantly influenced by co-administration of conjugate 1, increase in the number of tumor-free mice was observed. The treatment with the dose eq. 7.5 mg Dox/kg of star-like pHPMA-Dox led to 25% of tumor-free mice on day 70 after tumor transplantation. The application of conjugate 1 fractioned to four doses increased the survival ratio to 62.5% of tumor-free mice at the same time, while the injection of a single dose of conjugate 1 increased the proportion of tumor-free mice to 50% (see Figure 4.14). As expected, an increase in the number of tumor-free mice was not seen when LMW Dox was combined with four doses of conjugate 1.

One mouse which received four doses of conjugate 1 together with star-like pHPMA-Dox died on day 98 with no sign of tumor growth at the site of tumor injection, but with excessively expanding lymph nodes. This expansion was most probably caused by the dissemination of tumor cells into the lymph nodes.

Furthermore, when conjugate 1 was used alone, a non-significant moderate decrease of median of survival by 3 days was observed (compared to control). The median of survival time during treatment by star-like pHPMA-Dox was increased by at least 20.5 days when conjugate 1 was administered in four doses and by at least 11.5 days

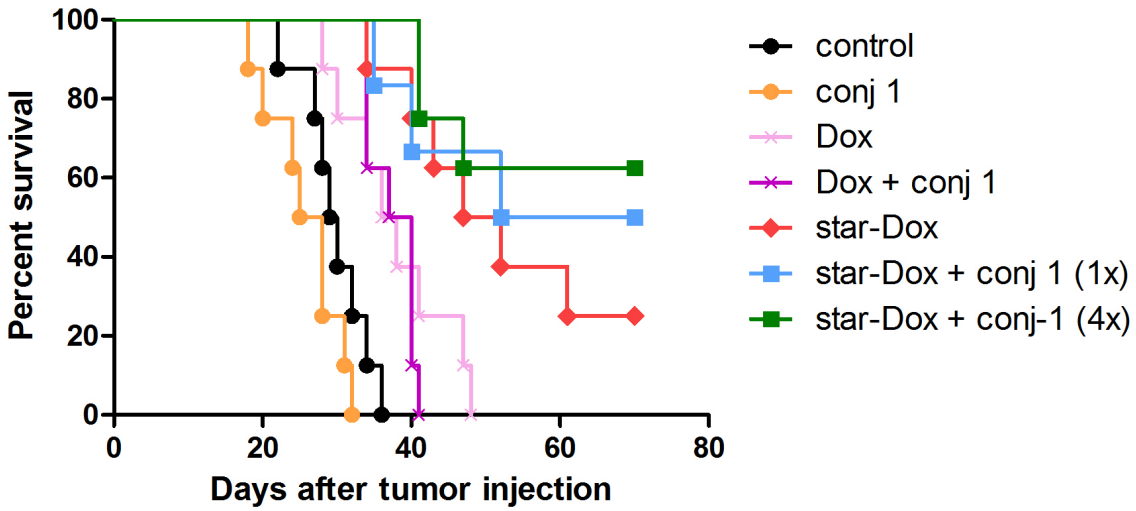

Figure 4.14: **Kaplan-Meier survival curves demonstrating influence of conjugate 1 on treatment outcome of star-like pHPMA-Dox treated C57BL/6 mice challenged with EL4 T lymphoma tumor.** Survival outcome of the experiment described in figure 4.13 was detected.

when a single dose of conjugate 1 was administered (see Table 4.2).

Previously, it had been thoroughly documented that after treatment with HPMA-based copolymers with cytostatic drugs, the mice that become tumor-free do not develop a tumor when they are challenged again by a lethal dose of tumor cells. The tumor resistance was observed in several tumor models when using HPMA-based copolymers with Dox or Dtx (Rihova *et al.*, 2010; Mrkvan *et al.*, 2005; Kovar *et al.*, 2008; Sirova *et al.*, 2007), Dtx and Ptx (Etrych *et al.*, 2010). The resistance towards another tumor challenge depends on the type of treatment and the dosage of drugs (Sirova *et al.*, 2007; Rihova *et al.*, 2009) and is tumor-specific.

The mice from the experiment showed in Figures 4.13 and 4.14 that survived and were tumor-free on day 100 were further challenged with a lethal dose of $1x10^5$ cells of EL4 T-cell lymphoma line. For retransplantation, two mice that received only star-like pHPMA-Dox treatment, four mice that received star-like pHPMA-Dox together with four doses of conjugate 1 and three mice that received one dose of conjugate 1 together with star-like pHPMA-Dox treatment were used. Six healthy mice were also challenged with the same dose of EL4 cells as a positive control for tumor growth.

The results showed that controls survived up to 27 days after the tumor challenge and only one mouse from the group treated by star-like pHPMA-Dox and four doses of conjugate 1 died on day 36 due to tumor development (see Figure 4.15). The growth

Treatment	**Median of survival (days)**	**Tumor-free mice (%)**
Control	29.5	0
Conj 1	26.5	0
Dox	37	0
Conj 1 (4 doses) + Dox	38.5	0
Star-Dox	49.5	25
Conj 1 (4 doses)+ Star-Dox	undefined	62.5
Conj 1 (1 dose) + Star-Dox	61	50

Table 4.2: **Survival of C57BL/6 mice treated with conjugate 1 and star-like pHPMA-Dox.** Median of days of survival of mice and ratio of tumor-free mice on day 70 after tumor injection from experiment in Figure 4.14.

of the tumor that this mouse was bearing was somewhat delayed when compared to control mice (see Figure 4.15).

Next, we tested the second polymer NO donor – conjugate 2 reproducing the same conditions as in the experiment with conjugate 1 described above and according to the same dosage scheme (see Figure 3.2).

Results showed that conjugate 2 alone did not influence tumor growth when compared to control. However, conjugate 2 slowed down the tumor progression when co-administered with star-like pHPMA-Dox. Four doses of conjugate 2 decelerated tumor progression less than a single dose (see Figure 4.16). This slowdown was, however, not significant and as progression of the tumor occurred only in one mouse within the group treated with a single dose of conjugate 2, no statistical analysis was applicable.

The treatment with the dose eq. 7.5 mg Dox/kg of star-like pHPMA-Dox led to 25% tumor-free mice on day 70 after tumor transplantation. The application of the conjugate 2 fractioned to four doses increased the survival proportion to 50% of tumor-free mice and injection of single dose of conjugate 2 increased the proportion of tumor-free mice to 87.5% at the same time (see Figure 4.17). The single dose of conjugate 2 significantly improved treatment of EL4 T-cell lymphoma by star-like pHPMA-Dox.

Conjugate 2 had a very similar change in median of survival as conjugate 1 — it caused 3.5 days-shorter survival when compared to untreated mice, but this effect was not statistically significant. Median survival during treatment by star-like pHPMA-Dox was increased by 19 days when conjugate 2 was administered in four doses, and

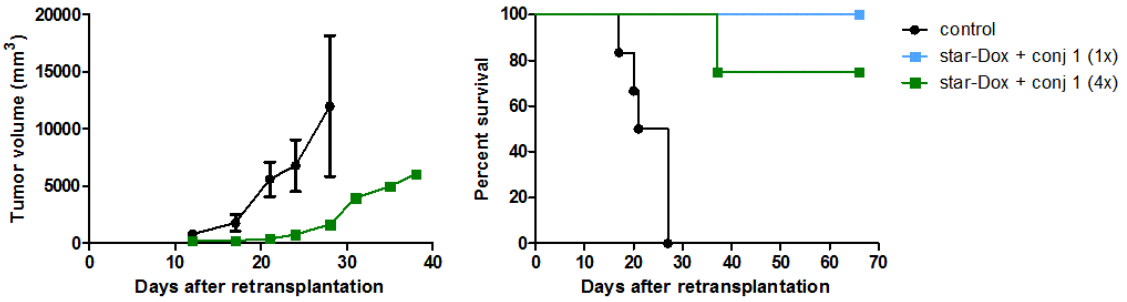

Figure 4.15: **Tumor growth (left) and Kaplan-Meier survival curve (right) demonstrating survival of mice retransplanted with EL4 cells after successful treatment by star-like pHPMA-Dox combined with conjugate 1.** The C57BL/6 mice that survived tumor-free after 100 days from experiment in Figure 4.14 were challenged with EL4 cells on day 103. Tumor volume mean ± SD values only from mice developing tumors are displayed.

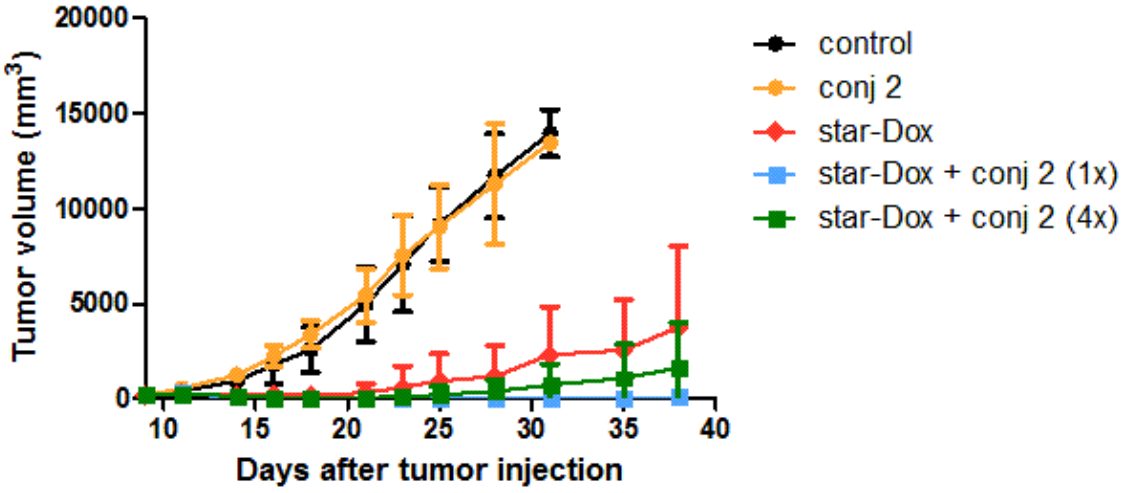

Figure 4.16: **Tumor growth of EL4 T lymphoma bearing C57BL/6 mice treated by star-like pHPMA-Dox and conjugate 2.** Four doses of conjugate 2 (20 µmol nitrate/kg each) at times -12, 0, 24 and 48 hours or 1 dose of conjugate 2 (80 µmol nitrate/kg) at time 0 hours respective to administration of star-like pHPMA-Dox conjugate (eq. 8 mg Dox/kg each) were administered i.v.. Tumor volume mean ± SD values are displayed.

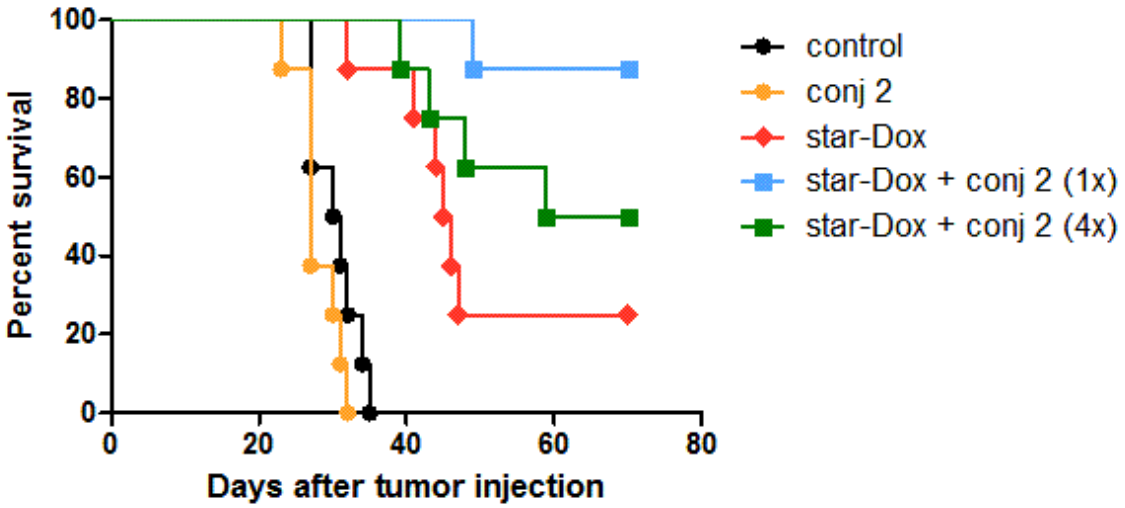

Figure 4.17: **Kaplan-Meier survival curves demonstrating influence of conjugate 2 on treatment outcome of star-like pHPMA-Dox treated C57BL/6 mice challenged with EL4 T lymphoma tumor.** Survival outcome of the experiment described in figure 4.16 was detected.

by at least 24.5 days when single dose of conjugate 2 was administered (see Table 4.2).

The retransplantation of conjugate 2 + star-like pHPMA-Dox cured mice by lethal dose of EL4 cells, same as in treatment with conjugate 1 is in progress.

Treatment	**Median of survival (days)**	**Tumor-free mice (%)**
Control	30.5	0
Conj 2	27	0
Star-Dox	45.5	25
Conj 2 (4 doses)+ Star-Dox	64.5	50
Conj 2 (1 dose) + Star-Dox	undefined	87.5

Table 4.3: **Survival of mice treated with conjugate 2 and star-like pHPMA-Dox.** Median of days of survival of mice and portion of tumor-free mice on day 70 after tumor injection from experiment in Figure 4.17.

4.7 Effect of Polymer NO Donors on Polymer-Based Chemotherapy Treatment of Murine 4T1 Breast Carcinoma

The experiments performed using the EL4 T-cell lymphoma model serve as a proof of concept, showing that the therapeutic effect of HMW polymer cytostatic drugs could be potentiated by their combination with a polymer NO donor. A sub-optimal dose of star-like pHPMA-Dox was used in these experiments, so that we could see potential improvement and potential worsening of the treatment outcome. An increase in dosage of the star-like pHPMA-Dox is still possible and could lead to the successful treatment of all the mice bearing EL4 T-cell lymphoma, with doses even smaller than MTD (Tomalova *et al.*, 2015). Therefore, the challenge lies in tumor models which are difficult to treat even with high doses of polymer-based treatment.

We decided to use a murine carcinoma model (4T1) on BALB/c mice to show whether the intensification of treatment outcome can be achieved. Based on our previous unpublished results, three doses of star-like pHPMA-Dox perform better then a single dose. Therefore, three doses of star-like pHPMA-Dox (each dose eq. 8 mg Dox/kg) were used and injection of the conjugate 1 was performed six hours prior to the administration of each dose of chemotherapy (see Figure 3.3).

Tumor growth was not significantly decelerated in any treated group, but the progression was slowed down slightly in treatment with star-like pHPMA-Dox, its combination with conjugate 1 and also when conjugate 1 was used as the only treatment (see Figure 4.18).

Although both treatments, either with star-like pHPMA-Dox or its combination with conjugate 1, moderately improved the survival rate when compared to untreated mice, the results showed that conjugate 1 extended the median survival of mice treated with star-like pHPMA-Dox only by 0.5 day (see Figure 4.19). Contrary to the EL4 model, when conjugate 1 was administered as the sole treatment, it did not negatively affect the survival median time. It even prolonged the median survival by 8.5 days (see Table 4.4), but the difference was not statistically significant when compared to the untreated control.

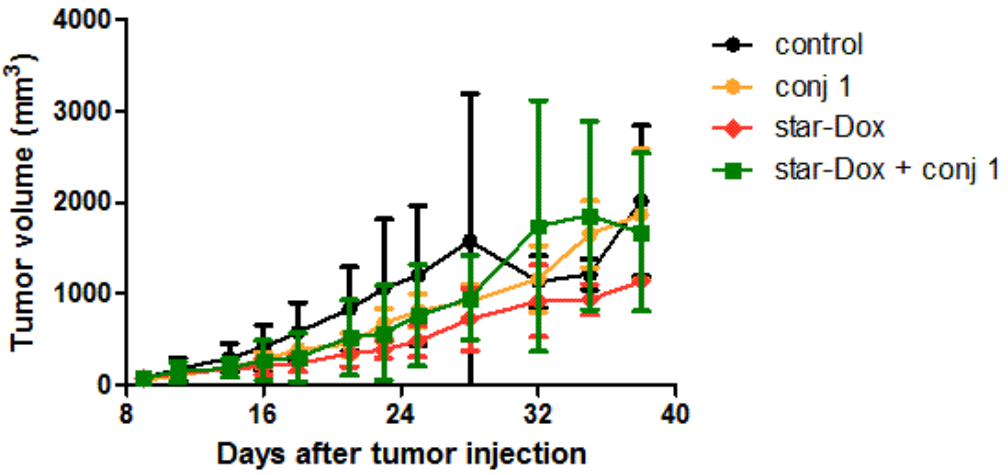

Figure 4.18: **Tumor growth of 4T1 breast carcinoma bearing BALB/c mice treated with star-like pHPMA-Dox and conjugate 1.** Three doses of conjugate 1 (20 µmol nitrate/kg each) at times -6 hours prior to administration of star-like pHPMA-Dox conjugate (each dose eq. 8 mg Dox/kg) on day 8, 11 and 14. All treatments were administered i.v.. Tumor volume mean ± SD values are displayed.

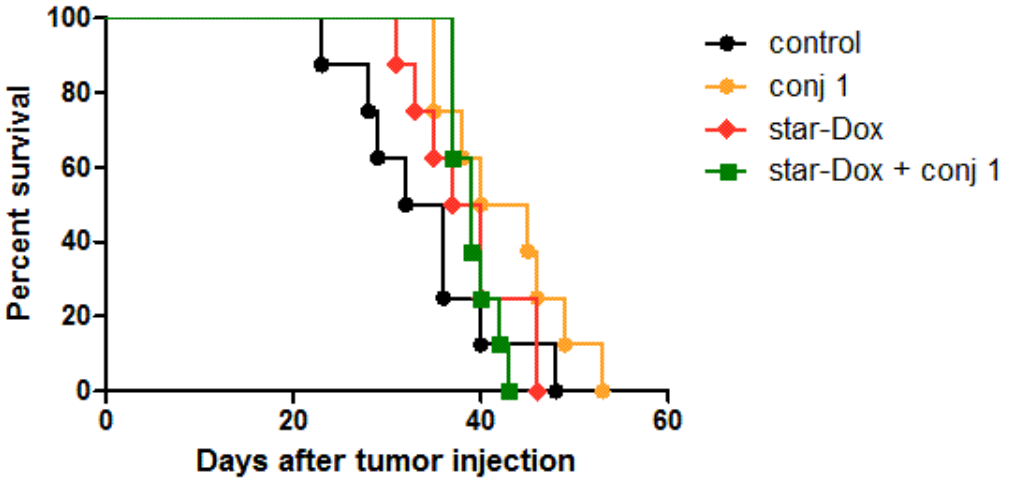

Figure 4.19: **Kaplan-Meier survival curves demonstrating effect of conjugate 1 on the treatment outcome of star-like pHPMA-Dox-treated BALB/c mice with 4T1 breast carcinoma tumor.** Survival time in the experiment described in figure 4.18 was monitored.

Treatment	Median of survival (days)
Control	34
Conj 1	42.5
Star-Dox	38.5
Conj 1 + Star-Dox	39

Table 4.4: **Median survival of BALB/c mice bearing 4T1 tumors treated with conjugate 1 and star-like pHPMA-Dox.** Median survival time of mice from the experiment showed in Figure 4.19.

4.8 Effect of Polymer NO Donors on DC Maturation

NO is a molecule with a high variety of functions and its role in innate and adaptive immunity (as described in Section 1.2) is very important. As the effective anti-tumor response is considered to be predominantly mediated by mechanisms of adaptive immunity, we wanted to look at the first line of this response — stimulation and maturation of dendritic cells. As mentioned in the Introduction, it is not well known what effect NO has on dendritic cells. There are contradictory results that need further examination.

First, we tested whether the polymer NO donors alone affect the expression of maturation markers on the $CD11c^{+}$MHC $II^{high}CD11b^{int}CD115^{-}$ BM-DC. The four tested concentrations of polymer NO donors and NG ranged from 0.02 to 20 µM. We used LPS (1 µg/ml) as positive control for the stimulation of the DCs.

The results showed that neither polymer NO donors nor NG affected the expression of maturation markers CD40, CD80 and CD86 (see Figures 4.20, 4.21 and 4.22).

Afterwards, we tested whether NG or polymer NO donors modulate stimulation of BM-DC. We incubated the BM-DC with LPS combined with the same four concentrations of polymer NO donors and NG ranging from 0.02 to 20 µM.

When NG was administered together with a stimulatory signal (LPS), the expression (evaluated as MFI) of the CD40 marker dropped significantly when compared to LPS-stimulated cells. Although the expression of CD80 was also slightly decreased, the difference was significant only in the case when 2 µM NG concentration was used. The CD86 expression showed a similar trend, but not a statistically significant decrease in expression when using higher concentrations of NG. The CD86 expression was slightly increased when the lowest concentration (0.02 µM) of NG was used (see Figures 4.20

and 4.21).

A similar effect was observed when using conjugate 1 together with LPS – there was a decrease in expression of the CD40 marker, but it was less apparent compared to the incubation with NG. Contrary to NG, conjugate 1 did not affect the expression of either CD80 or CD86 (see Figures 4.20 and 4.22). Interestingly, the decrease in CD40 expression after stimulation with LPS and both NG or conjugate 1 was more significant when lower concentrations of NO donors were used.

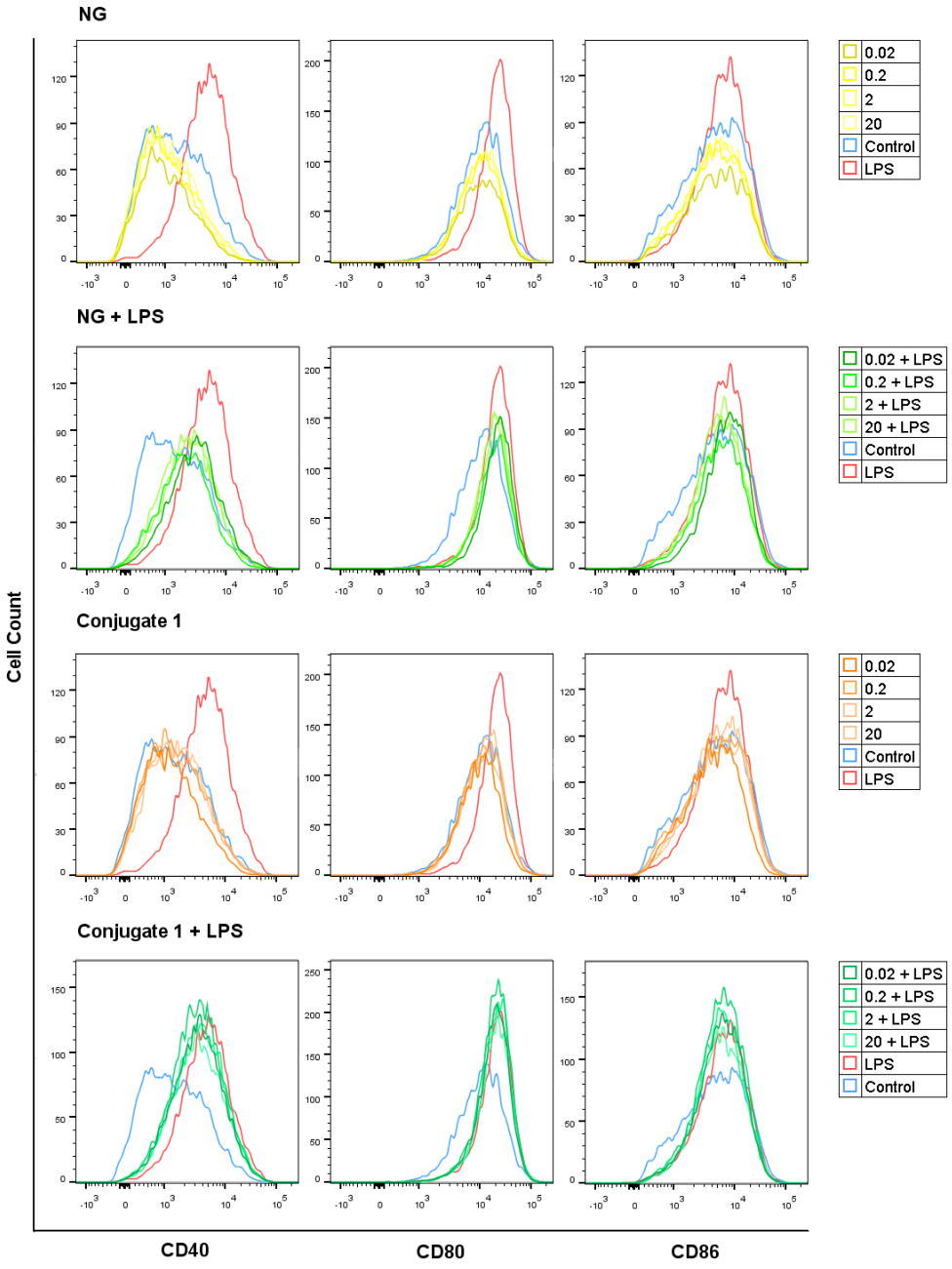

Figure 4.20: **Effect of NG and conjugate 1 on stimulation and expression of maturation markers CD40, CD80 and CD86 on BM-DC.** Maturation markers were detected on CD11c^{+}MHC II^{high}CD11b^{int}CD115^{-} BM-DC generated from C57BL/6 mice using rmGM-CSF and IL-4. The membrane expression of the markers was detected by LSR II flow cytometer and analyzed using FlowJo software. First, the BM-DC were incubated with four different concentrations (displayed as µM nitrate) of NG or conjugate 1, and LPS (1 µg/ml) was used as positive control (first and third row). Next, the BM-DC were stimulated by combination of the same concentrations of conjugate 1 or NG together with LPS (second and fourth row). Overlay histograms of CD40, CD80 and CD86 expression are displayed. Results of one representative experiment from three independent experiments are displayed.

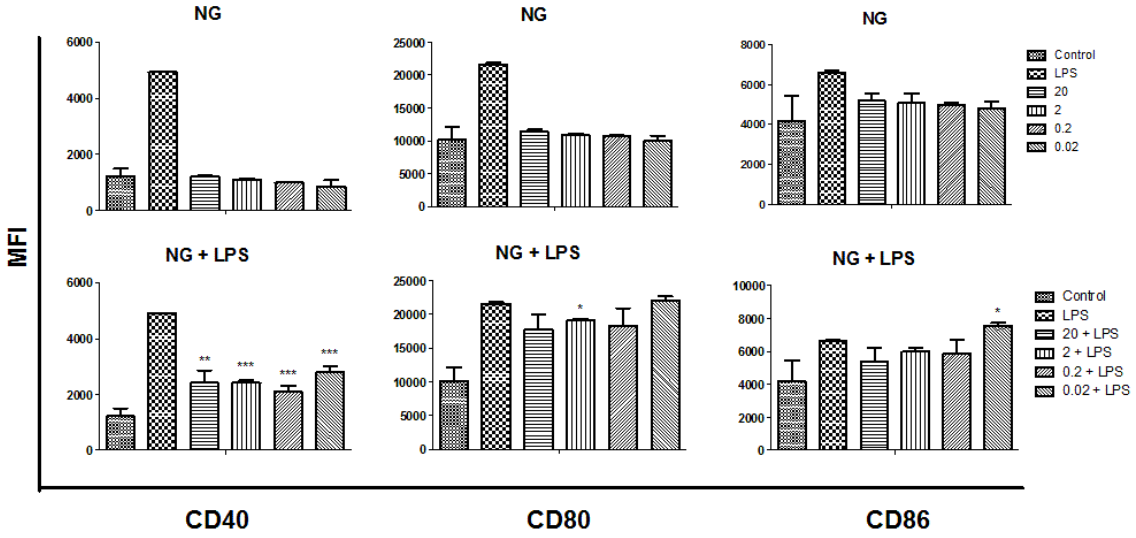

Figure 4.21: **Mean fluorescence intensity of maturation markers CD40, CD80 and CD86 on BM-DC stimulated by NG and LPS.** MFI of maturation markers on $CD11c^{+}$MHC $II^{high}CD11b^{int}CD115^{-}$ BM-DC were as depicted in the Figure 4.20 are shown. Upper row represents BM-DC treated by NG, bottom row represents BM-DC treated by both NG and LPS.

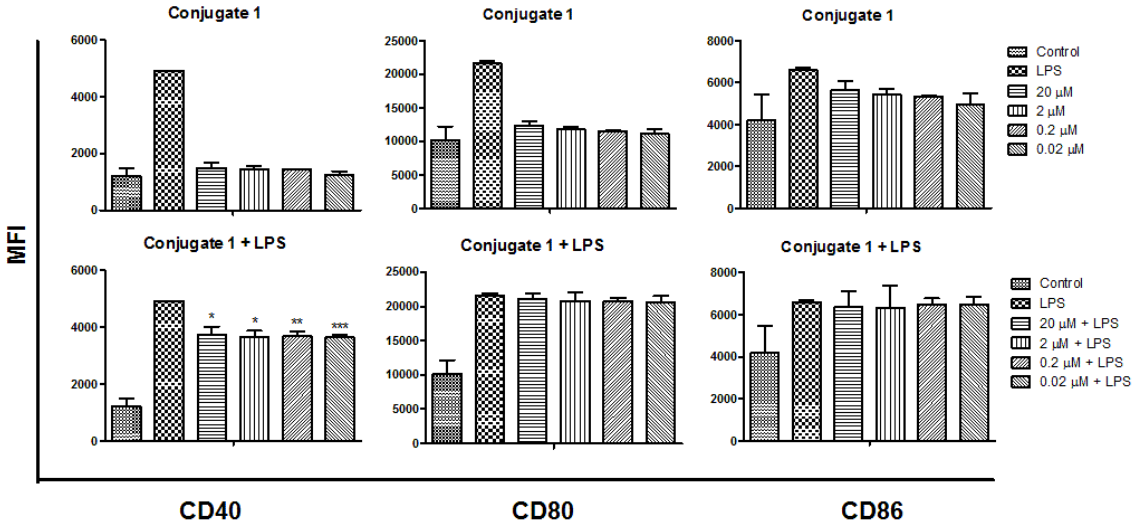

Figure 4.22: **Mean fluorescence intensity of maturation markers CD40, CD80 and CD86 on BM-DC stimulated by conjugate 1 and LPS.** MFI of maturation markers on $CD11c^{+}$MHC $II^{high}CD11b^{int}CD115^{-}$ BM-DC were as depicted in the Figure 4.20 are shown. Upper row represents BM-DC treated by conjugate 1, bottom row represents BM-DC treated by both conjugate 1 and LPS.

5. Discussion

Current tumor treatment approaches are often effective, but they are accompanied by a variety of side effects. One of the strategies for the elimination of such side effects is the usage of polymer carriers that allow preferential accumulation in tumors, e.g. HPMA-based copolymers. Even treatment with polymer-based cytostatic drugs is not efficient enough for some types of tumors. Augmentation of the EPR effect provides one of potential strategies for further intensification of the polymer treatment effect. Such augmentation may be achieved using vasodilatory substances, which can release NO that intermediates smooth muscle relaxation and vasodilation (Seki *et al.*, 2009).

Using LMW NO donors poses several problems. Their short half-life and rapid biotransformation cause systemic side effects which may lead to hypotension, which can result in tissue damage or even death – a similar phenomenon to the one that occurs during sepsis (Titheradge, 1999). Indeed, they have no no selectivity towards tumor cells or tissue. Administration of LMW NO donors is therefore usually topic (Seki *et al.*, 2009) and this does not allow for precise dosage control. Moreover, local treatment only enables treatment of cutaneous or subcutaneous tumors.

Many NO donors e.g. NO-releasing nanoparticles (Stevens *et al.*, 2010) or DETA-NONOate (Pervin *et al.*, 2001) have cytotoxic or cytostatic activity towards the cancer cells. We have shown that HPMA-based polymer NO donors do not exert such toxicity. They are non-toxic to the permanent cancer cells *in vitro* in a wide range of concentrations. Therefore we suppose that only an indirect effect (enhancement of drug accumulation in tumor through vasodilation) will occur during the application of the polymer NO donors in treatment of experimental murine tumors.

In the *in vitro* tests of conjugates 1 to 3 and NG, cell cultivation conditions had to be modified because gluthatione in RPMI may react spontaneously with organic nitrates, resulting in nitrite formation (Heppel L. A., 1950). That could affect the functionality of the polymer NO donors. The toxicity of the polymer NO donors to primary cells could not be assessed because the murine splenocytes did not proliferate in D-MEM in the presence of mitogens (e.g. concanavalin A), which otherwise potently stimulate the cells in RPMI. Considering that polymer NO donors do not affect proliferation activity of the permanent cell lines, we presume that toxicity towards normal cells does not occur.

Chemosensitization of several types of the tumor cells towards chemotherapy and radiotherapy using NO donors has been described (Evig *et al.*, 2004; Frederiksen

et al., 2007; De Boo *et al.*, 2009; Ning *et al.*, 2014). The possible mechanisms of chemosensitization have been reviewed by Sullivan & Graham (2008). We tested whether the polymer NO donors or NG can cause such sensitization. We examined a wide range of concentrations and studied their influence on Dox and Dtx cytostatic activity in EL4.IL-2 and 4T1 cells. We could not detect any influence on cytostatic activity, not even with LMW NG. The same mechanism was also tested in the epithelial hybridoma model EA.hy926 with no effect of NO donors on the cytostatic activity of Dox or Dtx. This suggests that the phenomenon of chemosensitization by NO donors is only applicable in certain cell types and cytotoxic drugs. Indeed, chemosensitization is not supposed to modulate the effect of star-like pHPMA-Dox treatment of EL4 T-cell lymphoma or 4T1 mammary carcinoma (see Section 4.2).

No toxicity of polymer NO donors was observed during the *in vivo* treatment of murine tumors. Healthy mice that obtained the highest tested dose of conjugate 1 and 2 eq to 150 µmol nitrate/kg did not show any signs of toxicity. This dose is much higher compared to the lethal dose of SNP, which is approximately 23 µmol nitrate/kg (Hospira Inc., 2011). The HPMA-based polymer NO donors bring the possibility of systemic delivery of higher doses of NO within the organism. There is a possibility that even higher doses than 150 µmol nitrate/kg can be still safe, but higher concentrations of the polymer NO donors were not tested.

It would be very beneficial to explore whether the administration of NO donors affects some parameters of the circulatory system, such as systemic blood pressure, but we do not have access to corresponding methods.

Although vasodilation is a common phenomenon in physiological regulation, prolonged exposition to vasodilatory substances, such as organic nitrates, has been described to invoke nitrate tolerance — the desensitization of vasodilatory mechanisms. The mechanism of such tolerance is complex and can be invoked by several pathways. Nitrate tolerance can have a different potency when different organic nitrates are used. These mechanisms are reviewed in Munzel *et al.* (2014). During tumor treatment, the administration of the polymer NO donors is not chronic, therefore the possibility of nitrate tolerance occurring is very low.

The vasodilatory properties of the selected polymer NO donor (conjugate 1) in tumors 4T1 and EL4 were examined. The effect of vasodilation — an increase of blood flow and potentially increased leakiness of the tumor — should lead to the intensification of the EPR effect. To determine such an effect, EB-albumin complex was used. This complex has been shown to work as macromolecules underlying the EPR effect in several types of tumor models (Matsumura & Maeda, 1986). However, in

our models this assay did not produce consistent results. That may have been caused by different characteristics, including capillary density and vascular permeability in the tumor, which varies throughout the tumor models and even in various sections of one tumor (Yuan *et al.*, 1995).

Although it has been shown that the EB-albumin complex is subjected to EPR effect (Matsumura & Maeda, 1986), it is still significantly smaller than star-like pHPMA-Dox conjugate that was used in our treatment. As the albumin-EB complex only has approximately 67 kDa, with an effective diameter of 7.2 nm (Kratz, 2008), we speculate that in our tumor models vasodilation does not necessarily have to improve its accumulation in tumor. However, as 250 kDa molecules with hydrodynamic diameter about 26 nm may be too large for some vascular pores, the vasodilation caused by NO release could enable much higher penetration of such molecules to the tumor interstitium, allowing for higher accumulation of the drug in the tumor.

To test whether the EB assay results are valid, we used direct detection of Dox content in tumor tissue samples following the application of star-like pHPMA-Dox conjugate. The accumulation of Dox in tumors was significantly increased after injection of star-like pHPMA-Dox together with a polymer NO donor, compared to treatment with sole HPMA-based copolymer with Dox. The increase was significant at 12, 24 and 72 hours after injection. Conjugate 1 also improved preferential accumulation of Dox in tumor samples when compared to Dox content in muscle samples. The content of Dox in liver samples was even higher than in tumor samples 12 hours after treatment injection, but preferential accumulation of Dox in tumors occurred later – after 24 and 72 hours. These results showed that the EB assay results do not correspond to actual accumulation of the drug in tumors.

As the accumulation of Dox released from star-like pHPMA-Dox was increased in EL4 tumors after treatment with a polymer NO donor (conjugate 1), we tested whether the actual treatment outcome is affected by co-administration of polymer NO donors. We tested two polymer NO donors (conjugate 1 and 2) in combination with star-like pHPMA-Dox in treatment of EL4 tumors. We used two dosage regimes — either four individual doses at time intervals -12, 0, 24 and 48 h respective to star-like pHPMA-Dox injection, or single dose equivalent to the sum of the smaller doses co-administered together with star-like pHPMA-Dox. Conjugate 1 did not change the polymer treatment effect on tumor growth when the four doses or single dose of the conjugate were used. Conjugate 2 decelerated tumor growth during polymer treatment — a single dose of the conjugate was more effective than four doses, but the difference was not statistically significant.

The influence on tumor growth was not determining, conjugate 1 actually improved the treatment outcome, expressed as a proportion of tumor-free mice on day 70 in the group, treatment by star-like pHPMA-Dox alone led to 2 tumor-free mice out of 8, while conjugate 1 and 2 co-administration improved the treatment outcome. Co-administration of four doses of conjugate 1 cured 5 mice out of 8, while one dose of conjugate 1 led to 4 tumor-free mice of 8 in the group. On the other hand, co-administration of four doses of conjugate 2 led to 4 tumor-free mice of 8, while the single dose of conjugate 2 caused a much larger improvement in the treatment outcome — 7 tumor-free mice out of 8. The application of polymer NO donors alone worsened median survival (moderately, but not significantly), similarly for both conjugates.

The difference in the treatment outcomes between conjugate 1 and 2 probably originates in the content of different organic nitrates. Their biotransformation mechanisms, and therefore effects, can differ, but the trend in NO release appears to be similar (Studenovsky *et al.*, manuscript in preparation). As they have approximatelly the same M_w, their pharmacokinetics should be similar. But pharmacodynamics may be different, as the organic nitrate on conjugate 2 is bound by hydrolytically labile bond, which possibly causes its faster transformation in a tumor microenvironment that may result in the improvement of the treatment outcome. However, more rapid transformation may also cause a small extent of NO release already in circulation, which may potentially affect blood flow and therefore increase the drug accumulation in the tumor. This effect would possibly lead to increased toxicity, but conjugate 2 did not show any observable systemic toxicity. Therefore, no rapid release of NO probably occurs in circulation. Direct determination of NO generation from the polymer NO donors and their stability in plasma or blood could not be performed, as no appropriate methods are available.

We consider the improvement of the treatment outcome caused by the co-administration of polymer NO donors remarkable. Undoubtedly, these results provide a proof of concept — polymer NO donors indeed mediate an increase in the EPR effect and are able to improve accumulation of macromolecular drugs in EL4 tumors. This intensification of the EPR effect really ameliorates the treatment outcome by polymer-bound drugs. However, we cannot exclude any additional effect that polymer NO donors may cause in a tumor microenvironment, e.g. influencing apoptosis or affecting induction of the immune response.

We further tested the polymer NO donor augmentation of EPR on a less-treatable model - murine 4T1 breast carcinoma. Although the application of four doses of polymer NO donor alone or together with polymer treatment decelerated tumor progression

when compared to control, the median of survival was almost unaffected. Surprisingly, a sole polymer NO donor caused the extension of median survival compared to untreated mice, but this effect was not statistically significant. There is a possibility that although the polymer NO donor may increase the accumulation of drugs in the tumor (not yet tested), the Dox level in the tumor still was not high enough to improve the treatment outcome.

As the treatment by polymer drugs based on HPMA cannot be successful without a functional immune response (Sirova *et al.*, 2007; Rihova *et al.*, 2009), we investigated whether polymer NO donors influence the maturation or stimulation of DCs.

Using BM-DC in the study of induction of adaptive immune response is a common approach. It has been known for a long time that such cells contain various sub-populations. However, only recently the $CD11c^{+}$ MHC II^{+} cells that had previously been considered to be DC, have been shown to comprise of both macrophages and DC. Stimulation of both these cell types may lead to a higher MHC II expression on both macrophages and DC, but they are different in terms of functional outcome (Helft *et al.*, 2015). The DCs generated from bone marrow precursors may also have a different ontogenetic origin and only part of them is derived from common dendritic cell precursor (Helft *et al.*, 2015). The BM-DC can be accessed as $CD11c^{+}$ MHC II^{high} $CD11b^{int}$ $CD115^{-}$ cells and their phenotype and functional response does not correspond to any special sub-population of DCs, but it combines characteristics of most of the DC subtypes known (Helft *et al.*, 2015). Therefore, we used this new assessment of BM-DC population in our experiments.

Upon incubation with polymer NO donors or NG, no effect on maturation markers CD80, CD86 and CD40 was observed in BM-DC. Here, LPS was used as positive control for stimulation. We further wanted to determine whether polymer NO donors or NG affect TLR-dependent (LPS) stimulation of DCs to any extent. Polymer NO donors did not affect the expression of markers CD80 and CD86 on LPS-maturated cells, however, a slight decrease in CD40 expression was observed.

A similar trend was detected when BM-DC were stimulated with LPS in the presence of NG or conjugate 1. NG decreased the CD40 expression much more than conjugate 1, and it even slightly (not significantly) decreased CD80 and CD86 expression when higher concentrations of NG were used. However, when a low concentration of NG was used, CD86 expression was even slightly increased.

Although it has been described that NO donors may influence BM-DC, which can lead to T-cell activation and proliferation (Perrotta *et al.*, 2004), the effect of NO donor on the expression of CD40 has not been described. CD40 is an important molecule for

the activation of APCs, B cells and Th1 cells. Further investigation of the functional effect of BM-DC treated by polymer NO donors needs to be conducted.

LPS stimulation has been shown to desensitize macrophages and monocytes towards CD40L activation via a cyclooxygenase-dependent mechanism (Sinistro *et al.*, 2007). It has been also shown that NO affects the activity of cyclooxygenase, which may suggest that LPS together with NO could possibly synergize in the desensitization of DCs towards CD40L activation, eventually reducing CD40 surface expression in DCs. The exploration of such pathway could bring better insight into LPS-stimulated activation of DC and the role of NO in this mechanism.

We can speculate that since immune response in tumor is mainly based on the cytotoxic effect of $CD8_+$ T cells, NO-induced down-regulation of the CD40 may potentially lead to a T_{h2} type of immune response. The change in CD40 expression is dose-dependent and it does not necessarily manifest *in vivo*. For a better understanding of NO influence on DC stimulation, functional effects on T-cell activation need to be examined first. Although LPS-stimulation of DC is an important mechanism, stimulation by the tumor antigens may result in a different expression pattern on DCs, and examination of the influence of NO on such stimulation would also be necessary.

6. Conclusions

- Although some NO donors have been shown to improve the effect of chemotherapy on cancer cells, we have determined that neither LMW organic nitrate NG nor organic nitrates bound to HPMA-based polymer carriers are capable of chemosensitization of murine EL4.IL-2, 4T1 and human EA.hy926 cells towards treatment with Dox or Dtx.

- We have demonstrated that polymer NO donors can lead to an intensification of the EPR effect. An increased accumulation of Dox in tumors caused by the application of a polymer NO donor was achieved during treatment of murine EL4 T-cell lymphoma with HMW star-like pHPMA-Dox conjugate.

- We have revealed that two polymer NO donors indeed significantly improved the treatment of experimental murine EL4 T-cell lymphoma with HMW HPMA-based copolymer with Dox. However, in a less-responsive tumor model — 4T1 breast carcinoma, only deceleration of tumor progression occurred.

- We have indicated that polymer NO donors alone did not cause maturation of BM-DC as detected by the expression of maturation markers CD40, CD80 and CD86. Their effect on LPS stimulation resulted in a decrease in CD40 expression. The LMW NO donor NG showed a more significant decrease in the expression of this marker. Such results indicate that polymer NO donors may potentially influence some mechanisms of immune response against tumors, but additional experiments are required.

7. References

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