I. INTRODUCTION

Cancer is a serious health problem worldwide. In economically developed countries, it is a second most frequent cause of death after cardiovascular diseases, and the number of oncological patients continuously increases with the increasing age of population.

The mainstay of cancer therapy is combination of surgery, radiation and chemotherapy. Whilst surgery and radiation are relatively precise and suitable to achieve a local control over the tumor, chemotherapy exerts a systemic effect. These three modalities, when properly combined and sequenced, can cure a substantial number of hematological cancers and a smaller, but still significant subset of various solid tumors.

Most cytostatic/cytotoxic drugs that are now in common use target the cells with high proliferation rate. The non-selective character of chemotherapy leads to increased toxicities towards normal rapidly proliferating cells. This means that the drugs have to be used at suboptimal doses, leading to development of (multi)drug resistance, metastatic disease and, eventually, to failure of the therapy. Innovative therapeutic strategies need to be developed in order to achieve better treatment outcome. For that purpose, several approaches are being applied. First, sophisticated genomic and proteomic research could identify appropriate tumor-specific molecular targets. Second, drug delivery systems (DDS) are being designed to bring the drug more precisely to the desired tissues and cells, and keep them away from the sites of potential damage, and/or to maintain the therapeutic concentration of the drug at the relevant sites for longer periods of time. Third, great effort is devoted to elaborate immune-based therapeutic strategies, harnessing the host anti-cancer immune responses.

I. 1. Targeted therapy of cancer

Targeted therapy is a term concerning with treatment strategies exploiting either molecular differences between cancer and normal cells („molecular targets“), or differences between healthy and cancer-associated tissues. The targeted therapies include several types of agents and strategies: (a) small molecules blocking enzymes specifically acting in cancer cells, (b) ligand-targeted therapies, and (c) high-molecular-weight (HMW) drug delivery systems.

I. 2. Low-molecular weight anticancer drugs

A representative of this class of drugs is imatinib mesylate (Gleevec/Glivec), a selective competitive inhibitor of BCR-ABL tyrosine kinase, which is constitutively active in chromic myeloid leukemia (CML) cells. The clinical use of imatinib significantly improved therapeutic outcome in patients with CML, as most of them (90 %) achieve a complete hematological remission [1]. However, mutations in

the *BCR-ABL* gene still occur in some patients, so that the tyrosine kinase is no longer being able to bind imatinib. New generation of inhibitors was developed; e.g. nilotinib and dasatinib are able to bind at least some of the mutated variants of the tyrosine kinase, and show improved clinical efficacy [2].

Other LMW agents target epidermal growth factor receptor (EGFR family), such as gefitinib, and erlotinib, both inhibiting the tyrosine-kinase active site of EGFR/ErbB1/Her1. Lapatinib is a dual inhibitor of EGFR/ErbB1/Her1 and ErbB2/Her2/Neu.

Unfortunately, in most cancers there are multiple abnormalities that should be targeted to achieve a clinically relevant therapeutic effect. It is extremely unlikely that drugs (such as imatinib mesylate) targeting a single (cancer-specific) gene product will be active against a major fraction of tumors.

I. 3. Ligand-targeted therapies

The ligand-targeted therapy or active targeting relies on differences in expression of the targeted molecules between cancer and normal cells. The ligand or antibody (i.e. targeting moiety) specifically binds the relevant structure on the cancer cell. Thus, ligand-targeted therapies could be a successful means of improving selective effect of anti-cancer drugs. Antibodies were the first targeting moieties utilized for these therapies, but other proteins, lectins, peptides, or aptamers are used as well.

I. 3. 1. Antibodies

A high number of antibodies specific either to "tumor-specific" antigens (expressed solely on cancer cells) or to "tumor-associated" antigens (normal cellular constituents that could be overexpressed or their expression is deregulated in cancer cells) was generated since monoclonal antibodies (mAb) were described by Köhler and Milstein [3].

Initial problems with immunogenicity of the murine mAbs in human patients (namely formation of human anti-mouse antibodies, HAMA), and limited ability of the murine mAbs to trigger Ab-dependent effector functions (such as antibody-dependent cellular cytotoxicity, ATCC, and complement-dependent cytotoxicity, CTC) were virtually solved by invention of chimeric, humanized, or fully human Abs [4]. More than 20 therapeutic antibodies are now approved for treatment, many of them for malignant diseases. Rituximab (anti-CD20), trastuzumab (anti-ErbB2/Her2/Neu), cetuximab (anti-), or bevacizumab (anti-VEGF) could be named as examples. An added advantage of using therapeutic mAb is a possible synergy between the antibody and chemotherapeutic agents, so that the cancer cells could be targeted in two independent ways.

Abs reacting with tumor-associated molecules could be conjugated or fused on the DNA level with a range of molecules to introduce additional functions.

These "armed" antibodies could show better therapeutic performances than their "naked" counterparts.

- Immunotoxins are conjugates composed of extremely potent toxins or toxin subunits linked to an internalizing antibody or other ligand (e.g. cytokine).
- Ab-drug conjugates (immunoconjugates) use (more potent) drugs that are bound to the Ab or Ab fragment
- Radioimmunotherapy employs conjugates of Ab with appropriate radionuclide to kill the target cells by radiation.
- Antibody-directed enzyme-prodrug therapy (ADEPT) is a two-step approach, in which an Ab-enzyme conjugate is first applied to localize the enzyme in the tumor, and LMW prodrug is then administered that is converted to an active drug by the activity of the targeted enzyme.

I. 4. High-molecular-weight delivery systems

These drug delivery systems are based on biochemical and physiological differences between tumor and normal tissue. Various delivery systems for conventional LMW cytotoxic drugs are being developed and tested. They either physically entrap their drug payload, or (more typically) the drug is covalently bound to a HMW carrier.

HMW delivery devices passively accumulate in the solid tumor tissue due to Enhanced Permeability and Retention (EPR) effect described by Matsumura and Maeda [5]. Tumor vasculature is more permeable for macromolecules than that in normal tissues. Moreover, there is very restricted or almost absent lymphatic drainage in the tumors. Thus, macromolecules can easily enter the interstitial space and accumulate there, because they are not dissipated by the defective lymphatic drainage. The EPR effect is molecular weight (Mw) and size-dependent.

I. 4. 1. Nanosized devices for passive drug targeting

A number of different HMW delivery strategies were developed, exploring either colloidal delivery vehicles, or water-soluble polymers as the drug carriers.

The most frequently used nanosized delivery devices are dendrimers, liposomes, emulsions and solid lipid nanospheres, micelles and other self-assembling nanoparticles, and polymer nanoparticles.

I. 4. 2. Water-soluble polymers as carriers for anti-cancer drugs

The conjugation of the drug to a HMW polymer carrier may offer several advantages:

- Provides water-solubility to the drug, even if the drug has a highly hydrophobic character. Therefore, better availability and a proper distribution is guaranteed

● Protects the drug at least temporarily from binding to serum proteins, enzymatic cleavage or scavenging processes. As a result, serum half-life and bioavailability of the drug are greatly extended, whilst renal clearance is minimized due to high Mw of the conjugate
● Polymer conjugates passively accumulate in the solid tumor tissue by EPR effect. Moreover, a targeting moiety could be incorporated into the conjugate that specifically binds the recognized molecule on the target cell.
● The conjugation significantly affects the whole body pharmacokinetics, resulting in better targeting of the conjugate to the desired site (tumor) and reduced access to normal tissues. This limits the systemic toxicity of the parent LMW drug.
● Drug conjugation also changes the fate of the drug at the cellular level. Many LMW drugs enter the cells rapidly (within minutes) by passage across the plasma membrane. The polymer conjugates are usually taken into cells more slowly by facilitated endocytic route [6]. The endocytosis of the drug at least partially bypasses the P-glycoprotein-mediated drug efflux what results in overcoming the (multi)drug resistance.

For the conjugation of drugs, both naturally occurring and synthetic polymers are utilized. The synthetic polymers offer some advantages over the biopolymers, such as tuneable composition and structure, possibility to introduce various functional groups to enable conjugation of drugs, and low or virtually absent immunogenicity.

The concept of using polymers as drug carriers was suggested already by Ringsdorf in 1975 [7]. A number of polymers were studied since then, carrying either proteins, or LMW cytotoxic drugs.

I. 4. 2. 1. Polymer-protein conjugates

Two main types of conjugates fall into this category: Pegylated proteins, and SMANCS.

● Pegylated proteins: The principle of conjugation of a protein with poly(ethylene glycol) (PEG) was proposed by Davis and Abuchowski in 1977 [8] since termed „pegylation". The conjugation of a protein with PEG (a) increases solubility and stability of the protein, and reduces its immunogenicity and antigenicity (b) prevents the rapid renal clearance of smaller proteins and receptor-mediated clearance of larger proteins by reticuloendothelial system, and (c) prolongs the plasma half-life. PEG itself is non-toxic and non-immunogenic.

Pegylation was applied to different peptides and proteins, namely enzymes or cytokines, and several are used in clinical practice (e.g. adenosine deaminase, L-asparaginase, interferons, erythropoietin, and colony-stimulating factors).

● SMANCS is a conjugate of neocarzinostatin (NCS), and poly(styrene-*co*-maleic acid) (SMA), named SMANCS [9,10]. Mw of SMANCS is 16 kDa,

but it binds non-covalently to albumin, thus *in vivo* behaves like a large protein of about 80 kDa. Compared to NCS, the conjugate showed several considerable pharmacological advantages, namely extension of biological half-life, enhanced tropism for tumor tissue, and decreased toxic side effects. SMANCS was approved in Japan for treatment of hepatocellular carcinoma [11].

I. 4. 2. 2. Polymer-drug conjugates

As with other HMW delivery systems, the clinical aim of polymer-drug conjugates is to achieve (a) improved drug targeting to the tumor tissue, (b) reduction of the unwanted toxicity by limiting access of the drug to the sites of toxicity, and (c) overcoming the mechanisms leading to drug resistance.

Most of these conjugates studied so far trade on anti-cancer drugs routinely used in clinical practice. Various types of polymers were tested, some of them are already approved for clinical use: PGA, poloxamer-188, poly(lactide-*co*-glycolide), PLGA).

I. 5. HPMA copolymer-bound drugs

N-(2-hydroxypropyl)methacrylamide (HPMA) is one of the most studied synthetic polymers used as drug carrier. The HPMA homopolymer was developed at the institute of Macromolecular Chemistry by Kopeček and co-workers [12,13]. Further development led to introduction of functional groups to the homopolymer, in order to enable conjugation of various drugs and later also targeting moieties [14]. GlyPheLeuGly (GFLG) spacer was designed to release the drug only intracellularly by the action of lysosomal proteases [15,16].

Preclinical tests proved biocompatibility of the HPMA homopolymer and copolymer (PHPMA), no inherent toxicity and absence of immunogenicity [17]. Similarly to PEG, binding of proteins to HPMA copolymer decreases the immunogenicity of the protein [18,19]. Studying of bio- and immunocompatibility of the HPMA-based conjugates has been associated with the name of B. Říhová since the very beginning – for review see [20].

The HPMA copolymer conjugates incorporated a number of LMW anticancer agents, such as chlorin e_6, ellipticin derivative, puromycin, wortmanin, geldanamycin, camptothecin, methotrexate and its derivatives, paclitaxel, 5-flourouracil, TNP-470, cyclosporin A, platinates, and several anthracyclines: epirubicin, daunorubicin, and doxorubicin.

The HPMA copolymer conjugates were prepared binding the drug either via enzymatically-cleavable bond [21,22], or via a pH-sensitive linkage [23,24]. Both types of conjugates are passively accumulated in solid tumor tissue by the EPR effect. Targeted HPMA copolymer conjugates were prepared, both binding the drug via amide bond, or via a hydrazone (pH-sensitive) linkage.

I. 5. 1. Non-targeted HPMA copolymer conjugates

The first conjugate in this class was a conjugate containing doxorubicin (Dox) bound via the enzymatically-cleavable bond through the GFLG linker (Dox^{AM}-PHPMA, also named PK1; FCE28068) [22]. The conjugate entered the clinical trial phase I in 1994 [25] and later on proceeded to phase II [26]. Its passive accumulation by EPR effect was documented [27], and some clinical activity in patients with non-small cell lung cancer (NSCLC), colorectal, and breast carcinoma.

HPMA copolymer conjugates containing other drugs, such as paclitaxel, camptothecin, or platinates, were also tested in clinical trials.

I. 5. 2. Targeted HPMA copolymer conjugates

HPMA-based copolymer conjugates have been specifically targeted by several targeting moieties, such as galactosamine, other ligands binding to the tumor cells (such as transferrin or folate), antibodies, and peptides.

Galactosamine-targeted conjugate entered the clinical trials phase I/II (also called PK2; FCE28069) as treatment for hepatocellular carcinoma (galactosamine binds to asialoglycoprotein receptor that is overexpressed on cancer cells, but is present also on normal hepatocytes). Higher accumulation of the conjugate in the tumor was not seen, and further clinical trials were abandoned.

II. AIMS OF THE STUDY

General aim of the study was to contribute to the development of HPMA-based polymer prodrugs for cancer treatment. This was divided into several specific aims and steps:

1. To contribute to the pilot clinical study in patients suffering from generalized breast cancer by testing of cytotoxic activity of natural killer cells

2. To examine anti-tumor efficacy of the Dox^{AM}-PHPMA conjugate containing human immunoglobulin (Dox^{AM}-PHPMA-HuIg), that was designed for the pilot clinical study, in an experimental murine tumor model

3. To contribute to the preclinical development of the Dox^{HYD}-PHPMA copolymer conjugate with pH-controlled release of the drug, namely to determine correlations between physico-chemical parameters and in vivo anti-tumor efficacy of the Dox^{HYD}-PHPMA conjugates

4. To evaluate *in vivo* anti-tumor efficacy of HPMA-based polymer prodrugs with pH-controlled activation containing paclitaxel and docetaxel

III. METHODS

All the studied conjugates were synthesized at the Institute of Macromolecular Chemistry, ASCR, v.v.i. , Laboratory of Biomedicinal Polymers.

The synthesis and characterization of the HPMA copolymer conjugates was described in several papers: for review see [28,29]. New types of HPMA-based conjugates containing paclitaxel (PTX) and docetaxel (DTX) as the bioactive agent are described in the last paper – thesis section III. 4.

Testing of anti-cancer effects of the HPMA copolymer conjugates was performed using syngeneic murine models of transplantable tumors. EL4 T cell lymphoma (C57BL/6, H-2b; both male and female mice), 4T1 mammary carcinoma (BALB/c, H-2d; female mice), 38C13 B cell lymphoma (C3H/He, H-2y; female mice) and BCL1 leukemia. (BALB/c, H-2d; female mice).

The pilot clinical study was performed starting 2000 in Prague, Faculty Hospital Motol. The patients signed an informed consent. Testing of the biochemical, hematological, and immunological markers during the treatment was done by an authorized laboratory, (Agilab, Prague.)

All methods in detail are described in the original papers which are part of the thesis.

IV. RESULTS AND DISCUSSION

IV. 1. Activation of immune mechanisms in patients treated with HPMA copolymer-bound doxorubicin conjugate containing human intravenous immunoglobulin

A conjugate consisting of a linear HPMA-based polymer backbone, to which drug (Dox) and human immunoglobulin (HuIg) are bound via a tetrapeptide GFLG linker (Dox^{AM}-PHPMA-HuIg), has been designed with the aim of using in human patients. The conjugate has high Mw (usually 500 to 1000 kDa) and thus it is passively targeted to solid tumors by EPR effect [5,30]. As an assumption, a small fraction of the conjugate could potentially be actively targeted, because the pool of human immunoglobulins (either autologous or human intravenous immunoglobulin) could contain antibodies reacting with antigens expressed on tumor cells. Experimental and clinical studies demonstrated that the intravenous immunoglobulin itself can exert beneficial therapeutic effect in cancer treatment.

The conjugate has been tested in a pilot clinical trial in Prague in patients with generalized breast cancer. However, similar HPMA-based conjugate containing epirubicin and autologous immunoglobulin was tested in a patient suffering from generalized angiosarcoma already in 1992.

The clinical study proved that the DoxAM-PHPMA-HuIg conjugate was stable during the circulation in the bloodstream, showed significantly prolonged circulation time, and was non-immunogenic. No antibody formation reacting with the conjugate of with the immunoglobulin itself [31] was observed. The administration of the conjugate was well tolerated even at repeated administrations (up to 5 doses) in all patients (9 so far). Partial clinical re-

sponse or stabilization of the disease lasting for months was recorded. Quality of life in all the patients considerably improved as a result of the treatment.

A broad array of hematological, biochemical and immunological parameters was monitored. Many tumor-related markers normalized during the treatment, and others improved. Increase in CD4+, CD8+, CD16+56+ cell numbers was recorded during the treatment course.

NK and lymphokine-activated killer (LAK) activity was tested using a functional cytotoxic assay, with K562 (NK sensitive), Raji and Daudi (LAK sensitive) cells as targets. We adopted the JAM test [32] in order to detect the NK and LAK activity in a small sample of full peripheral blood. Increase of NK and LAK activity triggered by the Dox-HPMA^{AM}-HuIg administration was detected in several patients, peaking on day 3 following the treatment. The finding is suggestive of activation of anti-cancer immune mechanisms [31].

The stimulatory effect of the Dox^{AM}-PHPMA-HuIg conjugate was marked by our previous finding that NK activity increased in athymic nude mice bearing human metastatic colorectal carcinoma SW 620 following 5 doses of non-targeted Dox^{AM}-PHPMA (PK1, FCE 28068) conjugate [33]. The PK1-treated mice had moderately retarded tumor growth and prolonged survival time relative to untreated controls, but the treatment obviously could not lead to a complete cure. In our later experiments with Dox^{AM}-PHPMA-HuIg conjugate, we documented the necessity of the intact immune system for complete tumor regression [34].

Publications:

Říhová B., Strohalm J., Prausová J., Kubáčková K., Jelínková M., Rozprimová L., Šírová M., Plocová D., Etrych T., Šubr V., Mrkvan T., Kovář M., Ulbrich K. (2003) Cytostatic and immunomobilizing activities of polymer-bound drugs: experimental and clinical data J. Control. Rel. 91: 1-16

Říhová B., Strohalm J., Kubáčková K., Jelínková M., Hovorka, O., Kovář, M., Plocová, D., Šírová, M., Šťastný M., Rozprimová L., Ulbrich K. (2002) Acquired and specific immunological mechanisms co-responsible for efficacy of polymer-bound drugs. J. Control. Rel. 78: 97-114

IV. 2. Anti-cancer effect of Dox^{AM}-PHPMA-HuIg conjugate in a model of murine T cell lymphoma

In order to obtain additional data on anti-cancer activity of the Dox^{AM}-PHPMA-HuIg conjugate, extensive investigation using several murine models was performed. We assumed that the main mechanism is a direct cytotoxic effect of the conjugate against cancer cells, facilitated by passive accumulation of the drug in the solid tumor due to the EPR effect [5,30].

In vitro, only a moderate cytostatic effect of the Dox^{AM}-PHPMA-HuIg was detected upon in EL4 lymphoma cells. Non-targeted conjugate DoxAM-PHPMA (PK1, FCE 28068) was rather less effective than the HuIg-containing conjugate. Free Dox inhibited the cell proliferation at a 3 orders lower concentration than the PHPMA conjugates, both with or without HuIg [34].

In contrast, the Dox^{AM}-PHPMA-HuIg conjugate proved to be highly effective in treating established EL4 tumors in immunocompetent C57BL/6 (B/6; H-2b) mice. A complete cure of EL4 tumors of was achieved in a substantial number of animals, depending on the dose and treatment regime. Further experiments exploring other murine syngeneic tumors, such as B16-F10 melanoma (B/6 mice; H-2b), 38C13 B cell lymphoma (C3H/N mice; H-2k), and 4T1 mammary carcinoma (Balb/c mice; H-2d) showed various efficacy of the Dox^{AM}-PHPMA-HuIg conjugate. In 38C13 B cell lymphoma and 4T1 mammary carcinoma, the efficacy was less prominent than in EL4 lymphoma, but we also achieved some complete tumor regressions [35]. A significant prolongation of survival time due to reduction of the tumor growth was found in B16-F10 melanoma, but no complete cure was recorded [34].

Complete cure was never seen in immunodeficient nude mice bearing EL4 lymphoma [34], or BCL1 leukemia [36]. The treatment-related tumor regression largely depends on the CD8+ tumor-specific T cells, as was shown by *in vivo* tumor neutralization (Winn's assay) [34].

The complete cure of mice induced by the Dox^{AM}-PHPMA-HuIg treatment is followed by development of long-term anti-tumor resistance in a significant proportion of the survivors. The resistant mice do not develop any tumor upon second challenge with a lethal dose of the same tumor cells without any therapy. The proportion of resistant animals inversely reflects the efficacy of the treatment [34]. The same relationship was documented when the EL4-bearing mice were treated with Dox conjugate with pH-controlled release of the drug [37], or with non-targeted Dox^{AM}-PHPMA (PK1) conjugate [38]. The resistance was also documented in other murine tumor models, such as BCL1 leukemia model using treatment with B1 mAb-targeted conjugate [36] and in mice cured from metastatic breast carcinoma 4T1 (Šírová, unpublished data). We show that the resistance is tumor-specific [34], and its development depends on the sufficient quantity of tumor-derived antigens [39]. The resistance was only documented in mice treated with PHPMA conjugates that do not impair the immune system.

Publication:

Šírová M. Strohalm J., Šubr V., Plocová D., Rossmann P., Mrkvan T., Ulbrich K., Říhová B. (2007) Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumour immunity in mice. Cancer Immunol. Immunother. 56: 35-47

IV. 3. Preclinical development of HPMA-based polymer therapeutics with pH-controlled release of Dox

Biological examinations showed striking differences in the sense of mechanisms of action when compared with conjugates binding the drug via amide bond. The pH-sensitive bond ensures fast release of the drug at low pH (pH 5-6). It means that Dox could be released from the polymer even extracellularly in the tumor interstitium (pH in the tumor microenvironment is lower than that in normal tissues), but release of the drug occurs predominantly intracellularly in late endosomes and lysosomes. It was proved that free Dox localized to nuclei. [40,41]. On the contrary, the release of the drug from the polymer backbone may not be a strict requirement in the HPMA copolymer conjugate in which the drug is bound via amide bond [42-44].

The conjugate with the hydrazone bond proved remarkably high cytotoxicity in a panel of tumor cell lines *in vitro* [24,45,29], importantly including those with limited content of lysosomes [46]. Considerable ant-tumor activity was demonstrated *in vivo* [24,47] in several murine models. Accumulation in tumor, prolonged circulation time and tumor-to-blood (T/B) ratio increasing with time imply that the conjugate passively accumulates in tumor by EPR effect.

In the first paper, we have shown that the Dox^{HYD}-PHPMA conjugate could induce even a complete eradication of established EL4 lymphoma tumors in a significant proportion of mice. A single dose of 75 mg Dox eq./kg injected between days 8 – 10 post tumor inoculation could induce a complete tumor regression in all the treated mice, and no side toxicity was recorded. Specific anti-cancer immune response (anti-tumor resistance) evolved in relation to the therapy. Again, we documented the inverse relationship between the tumor treatment efficacy and development of tumor resistance.

In the second publication, modification by adding positively or negatively charged chemical groups or a hydrophobic substituent (oleoyl group) to the side chains of the polymer is described [48]. The aim was to achieve conjugates with higher hydrodynamic radii and thus higher accumulation in the tumor tissue. The presence of charged groups could cause an increase of the hydrodynamic radius either by swelling of conjugate coils due to electrostatic repulsions or by self-assembly of shorter polymers into polymer micelles. The introduction of the charged groups (less than ~ 8 mol %) was probably too small to significantly increase the hydrodynamic radius, whilst the conjugate containing the oleoyl groups formed stable micelles with a narrow size distribution.

All the conjugates demonstrated significantly higher therapeutic efficacy than free Dox at any dosage tested. The conjugate containing negatively charged GFLG sequences had moderately better anti-tumor activity *in vivo* in the EL4 lymphoma model than the unmodified Dox^{HYD}-PHPMA conjugate.

Side toxicity was seen in the conjugate forming micelles (i.e. conjugate containing oleoyl groups) at a high dose (2×25 mg Dox eq./kg), and was still apparent at a low dose (1×15 mg Dox eq./kg).

Another series of conjugates was prepared containing hydrophobic substituents, such as dodecyl, oleoyl, and cholesteryl [49]. The conjugated formed micelles or stable associates with high hydrodynamic radii (RH up to18 nm) in water, thus improved passive accumulation due to the EPR effect was supposed [5]. Conjugates substituted with cholesteryl groups formed self-assembling supramolecular structures with the highest RH (9–18 nm). High anti-tumor effect was produced by the conjugate bearing cholesteryl groups even at lower dosages (1×10 and 2×5 mg Dox eq./kg). Tumor resistance developed in the cured mice similarly as was previously shown in the linear Dox^{HYD}-PHPMA conjugate. The efficacy of the cholesteryl-substituted conjugate was higher than that of the Dox^{HYD}-PHPMA conjugate without substitution, the conjugate had slower blood clearance than the linear one, and also showed increased accumulation than the linear conjugate.

Another HPMA copolymer suitable for enhanced passive accumulation in tumor is described [50], formed by HPMA copolymer backbone grafted with similar but semitelechelic HPMA copolymer chains. The copolymer chains bind Dox via the pH-sensitive hydrazone bond. Accentuated accumulation by EPR effect was shown in this conjugate, as well as increased in vivo antitumor efficacy in 38C13 B cell lymphoma and EL4 T cell lymphoma models.

In the last publication, preclinical evaluation of the linear HPMA-based copolymer conjugate with pH sensitive release of Dox in murine models is described [51]. We have tested conjugates with different content of the bound drug (Dox). It is an important parameter in in relation to the efficacy of the treatment. The best anti-tumor effects were produced with the conjugates containing ~ 10 wt% of the bound Dox. The conjugates with higher drug content showed decreased in vivo efficacy. The low in vivo efficacy of conjugates with the high drug amount (16–22 wt%) could be explained by lower in vivo biavailability of the drug, resulting from the more hydrophobic character of the conjugate.

We also showed that free doxorubicin, that is regularly present in the conjugate preparations (usually less than 0.2 wt% relative to total drug content) is by far safe, and content of the free dug up to 4.6 % had no impact on the efficacy of the treatment and acute toxicity. Indeed, the conjugates induced complete cure of the treated animals, followed by inductin of anti-tumor resistance. No myelosuppression or organ damage was observed even at high dose of the Dox^{HYD}-PHPMA- conjugate (75 mg Dox eq./kg).

Publications:

Mrkvan T., Šírová M., Etrych T, Chytil P., Strohalm J., Plocová D., Ulbrich K., Říhová B. (2005) Chemotherapy based on HPMA copolymer conjugates with pH-controlled release of doxorubicin triggers anti-tumor immunity. J. Control. Rel. 110: 119-129

Chytil P., Etrych T., Koňák Č., Šírová M., Mrkvan T., Říhová B., Ulbrich K. (2006) Properties of copolymer-doxorubicin conjugates with pH-controlled activation. Effect of polymer chain modification. J. Control. Rel. 115: 26-36

Chytil P., Etrych T., Koňák Č., Šírová M., Mrkvan T., Bouček J. Říhová B., Ulbrich K. (2008) New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting. J. Control. Rel. 127: 121-130

Etrych T., Chytil P., Mrkvan T., Šírová M., Říhová B., Ulbrich K. (2008) Conjugates of doxorubicin with graft HPMA copolymers for passive tumor targeting. J. Control. Rel. 132: 184-92

Šírová M., Mrkvan T., Etrych T., Chytil P., Rossmann P., Ibrahimová M., Kovář L., Ulbrich K., Říhová B. (2010) Preclinical evaluation of linear HPMA-doxorubicin conjugates with pH-sensitive drug release: anti-tumor efficacy, safety, and immunomodulating activity in murine model. Pharm. Res. 27: 200-208

IV. 4. Anti-cancer activity of polymer prodrugs with pH-controlled drug release, containing paclitaxel or docetaxel

Taxanes, such as paclitaxel PTX) and docetaxel (DTX) are modern anti-cancer agents that demonstrated their clinical efficacy in the treatment of various solid tumors and some hematological malignancies. The main drawback in their practical utility is their very low water solubility, and, indeed, systemic toxicity that is otherwise typical for LMW anti-cancer drugs. Several HMW delivery systems developed over time, such as liposomes, nanoparticles, or polymer prodrugs, overcome the problem of low water solubility [52,53]. However, most of these formulations showed limited anti-cancer efficacy or induced serious side effects. Paclitaxel prepared in the form of nanoparticles with albumin outer shell received its approval for treatment of metastatic breast cancer as Abraxane (ABI-007) in 2006 [54].

We describe synthesis, characterization and results of preliminary biological testing of conjugates containing PTX or DTX. The anti-cancer efficacy was

tested in two syngeneic tumor models: EL4 lymphoma, and 4T1 mammary carcinoma. The 4T1 model is considered a model of human breast cancer disease, characterized by low immunogenicity and modest curability. Similarly as HPMA copolymer conjugates with Dox bound via pH-sensitive bond, the conjugates containing PTX or DTX demonstrated significant anti-tumor activity in vivo. Their treatment effect highly out-performed that attainable with the free drug. Moreover, no side toxicity was recorded. Most notably, PTX and DTX-containing conjugates induced complete tumor regression together with development of anti-tumor resistance, the valuable characteristic of Dox-containing HPMA-based conjugates.

V. CONCLUSIONS

1. We used HPMA copolymer-bound doxorubicin conjugate containing autologous or human intravenous immunoglobulin for treatment of patients with generalized breast cancer, who were refractory to conventional treatment. The administration of the conjugate was well tolerated in all the patients. A considerable proportion of biochemical and immunological markers having pathological values before the treatment normalized or improved as a result of the therapy. Clinical improvement was recorded, and quality of life of all the treated patients was very good.

Administration of Dox^{AM}-PHPMA-HuIg conjugate to the patients led to treatment-dependent increase in NK and LAK activity in peripheral blood. Human intravenous immunoglobulin proved its applicability for passive/active targeting of the HPMA-based copolymer conjugate in human patients for treatment of solid tumors.

Enhancement of the NK activity detected in peripheral blood supports the possibility of activation of anti-tumor immune responses induced by the treatment with DoxAM-PHPMA-HuIg.

2. Dox^{AM}-PHPMA-HuIg conjugate exerts substantial anti-tumor effect in several murine tumor models. The conjugate can induce a complete tumor regression of established solid tumors, depending on the treatment dose, and regime. An intact immune system is a prerequisite for the complete tumor regression. Long-term tumor-specific resistance develops in immunocompetent mice, which is related to the treatment dose and efficacy. This dual anti-cancer activity, i.e. the direct anti-cancer efficacy together with the induction of tumor resistance, is probably the most important feature of the Dox^{AM}-PHPMA-HuIg treatment.

The conjugate represents a potential cancer treatment for a variety of solid tumors. It is effective, safe, devoid of most side effects characteristic for LMW cytotoxic drugs, and endowed by immunomodulatory effects further enhancing its activity.

3. The preclinical study of biological characteristics of HPMA copolymer conjugates with the drug bound via pH-sensitive bond illustrates that efficient polymer conjugates are products of a rational design. Parameters such as content of the bound drug, Mw of the conjugate, substitution of side chains by various substituents, and the structure and stability of the bond that binds the drug to the polymer backbone are key parameters determining the physicochemical characteristics, and subsequently biological activity of the conjugate. A relatively simple synthesis of the linear Dox^{HYD}-PHPMA conjugate and its remarkable therapeutic safety, efficacy, and ability to induce a complete tumor regression of established tumors made the conjugate a promising candidate for clinical trials.
4. Binding of hydrophobic agents such as PTX and DTX to HPMA-based polymer backbone via pH-sensitive hydrazone bond produced conjugates with excellent water solubility. These conjugates showed convincing anti-tumor capacity in murine models and induced a complete regression of established EL4 lymphoma or 4T1 mammary carcinoma tumors. The treatment-related tumor resistance developed in a significant proportion of the cured animals both in EL4 lymphoma and 4T1 carcinoma.

The results substantiate the remarkable safety, effectiveness, and immunomodulatory capacity previously demonstrated in several HPMA-based conjugates containing Dox as the bioactive agent. This again points to the high prospective clinical applicability of the HPMA copolymer conjugates in treatment of solid tumors.

VI. POUŽITÁ LITERATURA/REFERENCES

1. Druker BJ, et al. (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med 355: 2408-2417.
2. Weisberg E, et al. (2007) Beneficial effects of combining nilotinib and imatinib in preclinical models of BCR-ABL+ leukemias. Blood 109: 2112-2120.
3. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.
4. Presta LG (2008) Molecular engineering and design of therapeutic antibodies. Curr Opin Immunol 20: 460-470.
5. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46: 6387-6392.
6. Duncan R, et al. (2005) Polymer-drug conjugates: towards a novel approach for the treatment of endrocine-related cancer. Endocr Relat Cancer 12 Suppl 1: S189-199.